Harnessing the immune system via FcγR function in immune therapy: a pathway to next-gen mAbs

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INTRODUCTION

The regulatory approval of the first therapeutic monoclonal antibodies (mAbs) in the 1980s ushered in the modern era of immune therapy. Since then, mAbs have become one of the most clinically successful therapeutic modalities across a diverse array of diseases. They have revolutionized the treatment of chronic inflammatory diseases and of some cancers including otherwise incurable malignancies.¹ They are commercially important and in 2017, five mAbs collectively grossed $45.6 billion in sales, placing them in the top ten drugs globally.² MAb development is expanding rapidly with over 100 mAbs approved for clinical use or in late-stage clinical trials and over 600 in various stages of clinical development.¹

The therapeutic actions of mAbs can take many forms—neutralization of the target such as cytokines in autoimmune disease, clearance of the target such as virus in infection or immunoglobulin (Ig)E in allergy, induction of innate effector cell activation that leads to target destruction by direct killing or the induction of apoptosis and the induction of adaptive...
immunity. Most therapeutic mAbs are IgG in origin and the heavy-chain subclass determines many of their biological properties including their long plasma half-life; complement activation, which is important in the action of some cytotoxic mAbs and importantly engagement by their fragment crystallizable (Fc) region with specific cell surface receptors, called FcR, the subject of this review.

In normal homeostatic immunity, there is a balance between IgG immune complex activation of proinflammatory responses through the activating-type FcRs—which leads to the destruction of opsonized pathogens—and of the modulation of these destructive effector responses by the inhibitory-type FcR, thereby avoiding injury to the host. Thus, therapeutic mAbs powerfully exploit these opposing activities, making them versatile drugs whose therapeutic potency can be improved by specific engineering of Fc–FcγR interactions.7

Many therapeutic mAbs depend, to varying degrees, on FcγR function (Figure 1, Table 1) for their mechanism of action (MOA) and/or their pharmacokinetic properties. For some mAbs interaction with FcγR is central to their MOA, such as the destruction of a target cell by antibody-dependent cell-mediated cytotoxicity (ADCC; Figure 1a) or antibody-dependent cell-mediated phagocytosis (phagocytosis or ADCP; Figure 1b). This also includes mAbs that may harness the inhibitory action of FcγRIIB to modulate the proinflammatory responses of immunoreceptor tyrosine activation motif (ITAM)-dependent receptor signaling complexes (Figure 1c). For other mAbs, FcγR may play a secondary role, such as the removal or “sweeping” of all immune complexes formed by cytokine or virus-specific neutralizing antibodies or of opsonized fragments of lysed target cells which in antigen-presenting cells may also feed the antigen into the antigen-presentation pathways (Figure 1d). In addition, FcγRs, particularly FcγRIIB (Figure 1e), are also key participants in the MOA of immune-stimulating agonistic mAbs or apoptotic mAbs by acting as a scaffold for the additional cross-linking of mAbs already bound to a cellular target, thereby inducing a signal in the target cell.

This review focuses on the cell-based effector functions that arise from the interaction of IgG with the classical human leukocyte FcγR.7 Although beyond the scope of this review, it should be noted that the IgG-Fc portion dictates other aspects of an antibody’s biology, including its serum half-life mediated by the neonatal FcR (FcRn),3 the activation of complement C1,8 antiviral protection via the intracellular receptor TRIM219 and interactions with the Fc receptor-like family.10

**HUMAN FcγR GENERAL PROPERTIES**

The human leukocyte receptors fall into two functional groups, namely, proinflammatory, activating-type receptors (FcγRI, FcγRIIa, FcγRIIc, FcγRIIa and FcγRIIb, which are also known as CD64, CD32a, CD32c, CD16a and CD16b, respectively) and the anti-inflammatory, inhibitory-receptor group (FcγRIIB also called CD32b) which was the first immune checkpoint described.

These FcγRs are high-avidity sensors of immune complexes which initiate, and then modulate, cell responses. In the context of normal immune physiology, opsonized target molecules can engage various FcγRs and induce a spectrum of effector responses which can be harnessed by many therapeutic mAbs (Figure 1, Table 1). These responses are not mutually exclusive and one therapeutic mAb may initiate various responses via different FcγRs and via different cell types.

Understanding the importance of cell-based effector functions in the MOA of therapeutic mAbs requires an appreciation of FcγR biology (Tables 1–3) which also underpins future efforts to tailor new mAbs for the exploitation-specific effector responses. In this review, we address only key aspects of the extensive knowledge of the human leukocyte FcγR family as it relates to effector functions. A number of other reviews more comprehensively explore FcγR biology, physiology, biochemistry, genetics and structure.7,11–14 Notwithstanding the recognized differences between the immunobiology of human FcγR and of rodents or nonhuman primates, animal models of FcR effector function in vivo have helped shape the strategies for the development of current therapeutic mAbs and are well reviewed.12,15 Furthermore, humanized FcγR models will provide even greater insights into the future.16

**FcγR expression on hemopoietic cells**

The tissue distribution of the human leukocyte FcγR is well documented and reviewed comprehensively elsewhere.7,11,17 In the context of effector functions harnessed by therapeutic mAbs, several aspects of the cellular distribution (Table 2) should be emphasized.

FcγR expression profiles differ between cell lineages but almost all mature human leukocytes, and platelets, express at least one FcγR (Table 2). It should also be appreciated that the cellular expression levels and receptor diversity as will be described later is also influenced by the activation state of the cells, anatomical location and the cytokine environment which modulates FcγR expression, particularly for FcγRI and FcγRIIB.16 For example, resting monocyte subpopulations may express only FcγRIIa but activated macrophages express FcγRI, FcγRIIa and FcγRIIa and/or FcγRIIB.7

Thus, specific characteristics of leukocyte FcγR expression are summarized as follows:

FcγRI is not usually expressed until induced by cytokines such as interferon-γ on monocytes, neutrophils, macrophages, microglial cells in the brain, dendritic cells
and mast cells. The sensitivity of FcγRI to interferon-γ suggests that its in vivo activity is closely tied to immune activation events, and mouse studies have suggested that it has a critical role early in immune responses.19,20 Its role in the MOA of antibodies may vary with anatomical location.21

FcγRIIa is expressed only in primates and shows the broadest expression of all FcγRs, being present on all innate

Figure 1. Graphical representation of the FcγR effector functions. (a) Natural killer cell antibody-dependent cell-mediated cytotoxicity via FcγRIIIa. (b) Antibody-dependent cell-mediated phagocytosis, and/or trogocytosis of large immune complexes, by professional phagocytes via activating FcγR such as FcγRIIIa and FcγRI; Biological sequelae include the destruction of the ingested complexes which may also feed antigen into antigen-presentation pathways of antigen-presenting cells (APCs). (c) Inhibition of cell activation by FcγRIIB. The immunoreceptor tyrosine activation motif (ITAM)-mediated signaling of B-cell antigen receptors (left) or of activating FcγR (right) on innate immune cells such as macrophages and basophils is inhibited by IgG Fc-mediated co-cross-linking of these activating receptors with the inhibitory FcγRIIB. This leads to phosphorylation of the FcγRIIB immunoreceptor tyrosine-based inhibitory motif (ITIM) and consequently recruits the phosphatases that modulate the ITAM-driven signaling responses leading to diminished cell responses. (d) Sweeping or internalization of small immune complexes leading to their removal and, in APC, to enhanced immune responses. (e) Scaffolding in which the FcγRs play a passive role. Typically involving FcγRIIB, no signal is generated in the effector cell but “super-cross-linking” of the opsonizing antibody by the FcγR on one cell generates a signal in the conjugated target cell, for example, induction of apoptosis or activation in agonistic expansion of cells and/or their secretion of cytokines. In extreme cases, this leads to life-threatening cytokine storm. ADCC, antibody-dependent cell-mediated cytotoxicity; Ag, antigen; BCR, B-cell receptor; Ig, immunoglobulin; NK, natural killer.

Table 1. FcγR responses relevant to therapeutic monoclonal antibodies (mAbs).

| FcγR-mediated mechanism of action | Effector responses | Action | Dominant receptor |
|----------------------------------|--------------------|--------|------------------|
| Activation                       | Antibody-dependent cell-mediated cytotoxicity | Direct killing of target cell | FcγRIIIa, FcγRIIa, FcRI |
|                                  | Antibody-dependent cell-mediated phagocytosis, trogocytosis | Direct killing of target cell | FcγRIIIa, FcγRIIa, FcRI |
|                                  | Antigen presentation | Vaccine-like immunity post-mAb therapy | FcγRIIIa, FcγRIIa, FcRI |
|                                  | Reduce B-cell proliferation or innate cell activation by antibody complexes | Inhibition of ITAM cell activation (i.e. BCR) or activating-type FcR (i.e. FcγR, FcεRI, FcαRI). Note that the FcγRIIB must be co-cross-linked with the ITAM activating receptor. | FcγRIIB |
| Sweeping                         | Internalization     | Removal of small immune complexes | FcγRIIIB |
| Scaffold                          | Target agonism or apoptosis | Passive “super-cross-linking” of mAb on opsonized target cell, for example, CD40, CD28, CD20, by FcγR on an adjacent cell | FcγRIIIB; also FcγRIIa, FcγRI |

BCR, B-cell receptor; ITAM, immunoreceptor tyrosine activation motif.

*Activating FcγR can also contribute to removal of complexes.
leukocytes. It is also present on platelets but its role in effector functions is not established but it is important in certain immune thrombocytopenias. A polymorphic form of this receptor is the only human receptor for human IgG2. This, together with its limited species expression and unique biology of IgG4 in these individuals. Its low frequency in the population may also confound its coexpression with FcγRIIa. The lack of FcγRIIib on macaque neutrophils appears to be compensated for by an increase in FcγRIIa expression.15

FcγRIIIs are the inhibitory-type FcγR and arise from a single gene. They lack intrinsic proinflammatory signaling and are instead immune checkpoints. They provide feedback regulation by antibodies, in the form of immune complexes, to inhibit B-cell activation by specific antigen. They also control activating-type FcγR function on innate cells. Two major splice variant forms of FcγRIIb exist with differential tissue expression profiles. FcγRIIb1 preferentially expressed on B lymphocytes contains a 20-amino acid cytoplasmic insertion necessary for membrane retention and cocapping with the B-cell antigen receptor (BCR). FcγRIIb2 is the predominant inhibitory receptor found on basophils and neutrophils, as well as on subpopulations of mast cells, dendritic cells and some monocytes/macrophages. FcγRIIb2 lacks the cytoplasmic insertion of FcγRIIb1 and consequently can internalize rapidly including with the activating FcR when they are co-cross-linked.11 It is not clear which form is present on human T cells.

One additional comment on tissue distribution is that FcγR expression on T cells has been difficult to establish unequivocally. However, there is increasing evidence that T-lymphocyte populations express FcγR. Some γδ T cells express FcγRIIia and αβ T cells reportedly express FcγRIIa, FcγRIIb or FcγRIIia but the significance with respect to effector function mediated by antibody is presently unclear.24-28

**Table 2. Properties of FcγR.**

| Receptor  | Affinity | IgG specificity          | Cell distribution                                                                 |
|-----------|----------|--------------------------|-----------------------------------------------------------------------------------|
| FcγRI     | High     | IgG1, IgG3, IgG4         | Induced by interferon-γ on monocytes, neutrophils, macrophages, dendritic cell subpopulations; mast cells |
| FcγRIla   | Low      | IgG1, IgG3, but IgG2 binding limited to the FcγRIlla-H131 form (~70% people) | All leukocytes and platelets except T and B lymphocytes                             |
| FcγRIIa   | Low      | IgG1, IgG3, IgG4         | NK cells                                                                          |
| FcγRIIb   | Low      | IgG1, IgG3               | Neutrophils                                                                       |
|           |          | Binding avidity reduced by Phe at position 158 | B lymphocytes, some monocytes (can be upregulated); basophils; eosinophils? Plasmacytoid and myeloid dendritic cells; NK cells only of individuals with FcγRIIb gene copy number variation |
|           |          |                          | Airway smooth muscle, LSEC, placenta, follicular dendritic cell                    |

Ig, immunoglobulin; NK cell, natural killer cell.

*Expressed in 20% of people.

Expression on nonhemopoietic cells

The immunobiology of FcγR is studied and understood almost exclusively in the context of hematopoietic cell
function but relatively recent investigations have identified and explored FcγR expression on nonhemopoietic cells. These studies suggest important roles in normal immune function and in the MOA of some therapeutic mAbs. The most extensively characterized receptor expression is FcγRIIb which is expressed on follicular dendritic cell, airway smooth muscle and liver endothelium. Its abundant expression on liver sinusoidal endothelial cells (LSECs) is estimated to represent the majority of in vivo FcγRIIb expression.17,29-31 As FcγRIIb lacks intrinsic proinflammatory signaling function, its role on these nonhemopoietic cells involves immune complex handling without the danger of, or the need for, induction of local tissue destructive inflammatory responses. On LSEC its major role appears to be immune complex sweeping, a process of removal of small immune complexes such as opsonized virus or macromolecules.17 This scavenging role by FcγRIIb on LSEC can be exploited in principle by mAbs forming small soluble complexes with their targets such as antiviral, anticytokine or similar antibodies.

**FcγR-DEPENDENT EFFECTOR RESPONSES**

**Not all opsonized targets are equal: size, distance, valency and Fc geometry affect potency**

To understand the immunobiology of FcγR effector responses particularly in the therapeutic mAb context, it is important to appreciate that the quality and potency of such effector responses is greatly affected by the nature of the IgG immune complex and/or the state of potential effector cells.

First, opsonization, per se, of a target is not necessarily sufficient to ensure FcγR interaction in a way that initiates an effector response. Although it is the IgG Fc that interacts with and clusters the FcγR to induce a response, the nature of the Fab interaction with its epitope can strongly influence the likelihood or potency of FcγR effector responses by influencing the density of appropriately presented Fc portions.35 and also the size of the immune complex.36 Furthermore, the display/orientation and geometry of the Fc portions, as a consequence of the fragment antigen-binding (Fab)
interaction with the target epitope, can result in effector responses such as ADCC that differ substantially in potency, presumably because of the orientation of the Fc makes FcγR engagement more, or less, accessible.37,38

Second, in innate effector cells at rest, the largely linear actin cytoskeleton and the extracellular glycosaminoglycan glycocalyx regulate function by interacting with large glycoproteins, such as CD44, arranging these into ordered “picket” fences.39,40 These corral receptors, including the FcγRs, and sterically inhibit their interaction with ligands. Upon cell activation, cytoskeletal remodeling is associated with the loss of the receptor corrales, allowing FcγRs and other receptors to freely diffuse, engage ligand, cluster and signal.39 The influence of such surface constraints on receptors and effector cell function helps explain some of the observed epitope distance requirements for optimal mAb function,39,41 which were apparent in a comparative study of ADCC and ADCP.42 ADCC was optimal when the epitope was displayed close, 0.3 nm “flush” or 1.5 nm, to the target membrane where close conjugation of effector and target by the mAb presumably facilitates the delivery of pore-forming proteins to the target membrane as required by ADCC. Interestingly, complement-dependent cytotoxicity which also utilizes pore-forming proteins for its cytotoxicity has similar distance constraints. By contrast, ADCP was poor when targeting epitopes displayed close or “flush” to the target cell membrane (within ~0.3 nm) but ADCP activity was restored when the epitope was displayed 1.5 nm off the membrane, demonstrating different optimal epitope distance requirements for ADCC and ADCP.42

Although the action of agonistic/antagonistic mAbs is mechanistically distinct to those eliciting cytotoxicity and ADCC, the distance segregation between target and FcγR⁺ cells is also important. Indeed, the membrane proximal epitopes of CD28 and CD40 are important for the FcγR function in the complex MOA of these mAbs.43,44

Clearly, the effects of immune complex valency, Fc density, presentation and geometry together with FcγR organization in the cell membrane suggest that the development of mAbs to certain targets will be heavily influenced by the context of use. Thus, improved mAb potency may not necessarily be achieved by engineering of the Fc polypeptide or its glycan alone. A more function-oriented approach early in mAb selection and development by, for example, application of rapid screening technologies that select for effector potency,34 followed by Fc engineering may be more productive.

ADCC and phagocytosis
ADCC and ADCP are the most widely appreciated FcγR-dependent effector functions (Figure 1a, b) and are, respectively, mediated primarily via FcγRIIa on NK cells and professional phagocytes such as macrophages. These effector functions, particularly NK cell ADCC, are believed to be major components of the MOA of cytotoxic therapeutic mAbs used in cancer therapy. In addition, ADCP can also occur via FcγRIIa and FcγRI,45 but the extent to which cytotoxic anticancer therapeutic mAbs depend on these for their MOA in patients is unclear. The improvement in clinical utility of mAbs engineered for selectively increased FcγRIIb binding suggests that FcγRIIa and FcγRI may be less important in vivo in cell killing effects but perhaps are more important in other aspects of therapeutic efficacy—discussed later.

Inhibition of cell activation by FcγRIIb
FcγRIIb is an immune checkpoint46,47 and its splice variants are potent modulators of ITAM-dependent signaling (Figure 1c). This modulatory function occurs only when FcγRIIb is coaggregated with an ITAM signaling receptor. Thus, B-cell activation by the binding of the antigen in the immune complex to the BCR is regulated by the simultaneous binding of the Fcs of the immune complex to FcγRIIb on the same cell. In innate leukocytes, the activating-type FcR (i.e. FcγRI, FcγRIIa, FcγRIIc, FcγRIII) and the high-affinity IgE receptor, FcεRI, and the IgA receptor, FcαRI, are all modulated by immune complex co-engagement with FcγRIIb2. The inhibitory function contributes to the MOA of therapeutic antibodies that target cell-activating molecules where the target cells also express the inhibitory FcγRIIbs such as the BCR (discussed later). Thus, B-cell activation is modulated by the simultaneous binding of the antigen in the immune complex to the BCR and the binding of the Fcs, also in the immune complex, to FcγRIIIB1 on the same cell.

Sweeping: clearance of small immune complexes
The removal of immune complexes in humans depends primarily on the complement receptor pathway and to a lesser degree the FcγR. Among the FcγRs, it has been widely believed that immune complex removal only occurs by phagocytosis/endocytosis of activating-type FcγR. Surprisingly, the inhibitory FcγRIIb, which lacks intrinsic activating function, plays a major role in clearance, and rapidly “sweeps” away small complexes from the circulation (Figure 1d).39,49 A major tissue involved in the clearance is likely to be the LSEC, where FcγRIIb is expressed abundantly in mice and humans. This role is potentially important in resistance to viruses and toxins but may also be key to optimal performance of therapeutic IgG mAbs whose primary MOA is believed
to be only neutralization of soluble macromolecules, for example, cytokines or IgE.

**FcγR uptake of antigen: antibody complexes and shaping the immune response**

Monoclonal antibody therapy is a form of passive immunization. Indeed, longer-term vaccine-like or vaccinal immunity has been demonstrated in anti-CD20-treated mice via FcγRIIa and in vitro recall memory responses from CD20-treated patients. Although this is dependent on FcγR and anti-CD20, the mechanism by which long-term anti-tumor response is established remains unclear.

Nonetheless, the active involvement of FcγR in the enhancement of antigen-specific immunity by uptake of immune complexes through FcγR is historically well documented in experimental systems where FcγRs bind immune complexes and thereby feed antigens into the antigen-presentation pathways. This has been demonstrated in vivo for small immune complexes via human FcγRI on human antigen-presenting cells and in mice. Similarly, the capacity of FcγRIIbs to bind and rapidly internalize antigen–antibody complexes suggests that it too may significantly influence feeding antigens into professional antigen-presenting cells of hematopoietic origin such as dendritic cells and possibly B lymphocytes.

Although not a classical major histocompatibility complex-dependent antigen presentation, FcγRIIb on the stroma-derived follicular dendritic cells influences antibody immunity by recycling antigen–antibody complexes to the cell surface for presentation of intact antigen to B cells.

Although somewhat speculative, FcγRIIb’s rapid internalization/sweeping of complexes by the abundant LSEC, which interact with lymphocytes and can present antigen, may have a significant role in shaping immune responses.

**Scaffolding of cell-bound mAbs by FcγR⁺ cells**

FcγR-expressing cells can be critical, but passive, participants in the MOA of some mAbs (Figure 1e). In FcγR scaffolding, IgG mAb molecules that have opsonized the cell surface of a target cell are additionally cross-linked by their Fc portions engaging the FcγRs that are arrayed on the surface of a second cell. This “super-cross-linking” of the target-bound mAb by the FcγR lattice or “scaffold” on the adjacent cell greatly exceeds the target cross-linking by the mAb alone, thereby inducing a response in the target cell. Scaffolding was originally identified as the basis of T-cell mitogenesis induced by anti-CD3 mAb. The CD3 mAbs alone were poor mitogens but the “super-cross-linking” of the T-cell-bound CD3 mAb by the membrane FcγR on adjacent cells, particularly by monocytes, induced rapid T-cell expansion and cytokine secretion but did not require activation of FcγR-expressing cells. Regrettably, FcγR scaffolding came to prominence and clinical relevance because of its causal role in the catastrophic adverse events resulting from the administration of anti-CD3 and anti-CD28 (TGN1412) mAbs.

Nonetheless, FcγR scaffold-based induction of intracellular responses in a target cell can also lead to beneficial therapeutic effects. Such examples are the induction of apoptotic death in a target cell, which is likely part of the MOA of daratumumab in multiple myeloma or the controlled agonistic expansion of cells, for example, via CD40 mAb agonism.

**IgG subclasses: specificity and affinity for FcγR**

Most FcγRs (Table 2) are weak, low-affinity receptors (affinities in the micromolar range) for IgG-Fc, irrespective of whether the IgG is uncomplexed, monomeric or when it is complexed with antigen (i.e. an immune complex). The very avid binding of immune complexes to an effector cell surface that displays an array of FcγR molecules is the result of the collective contributions of the low-affinity interactions of each Fc of the IgGs in the complex with an FcγR. This avidity effect is necessary as the FcγRs operate in vivo in environments of high concentrations of uncomplexed monomeric IgG (normally 3–12 g L⁻¹). Thus, the avid multivalent binding of the complex outcompetes uncomplexed, monomeric IgG. The notable exception to this is the enigmatic FcγRI. This receptor shows high, nanomolar affinity for uncomplexed monomeric IgG and thus would be expected to be constantly occupied in vivo by the normal circulating monomeric IgG. However, IgG dissociation permits engagement with immune complexes. Furthermore, FcγRI is not expressed or expressed poorly on resting cells, requiring interferon-γ for induction of its expression, presumably at sites of inflammation.

Although the human IgG heavy-chain constant domains have greater than 90% identity, key amino acid differences confer each subclass with unique structural and functional properties. IgG1 and IgG3 are “universal” ligands, that is, they bind to all FcγRs. Formal measurement of the weak, micromolar Kᵢ, interactions of the low-affinity receptors with monomeric IgG1 also revealed differing affinities between the low-affinity FcγRs, with inhibitory FcγRIib generally having the lowest affinity and FcγRIII the higher, sometimes referred to as a “moderate” affinity receptor.
The strength of IgG1 interaction can also be affected by FcγR polymorphism and in the context of therapeutic mAbs, variation in FcγRIIIa is particularly important. The most common and possibly clinically significant polymorphism is phenylalanine/valine variation at position 158 in the IgG-binding site, wherein FcγRIIIa-F158 binds IgG1 less well than the FcγRIIIa-V158 form.

IgG4 and IgG2 have more restricted FcγR specificity. IgG4 has low affinity (K_A = ~2 × 10^5 M^-1) for the inhibitory FcγRIIib, but is also a high-affinity ligand for FcγRI (K_A = ~4 × 10^8 M^-1). IgG2 exhibits a highly restricted specificity, showing functional activity with only one polymorphic form of FcγRIIa (binding affinity K_A = ~4.5 × 10^5 M^-1) which is permitted by the presence of histidine at position 131 of its IgG-binding site. This FcγRIIa-H131 form is expressed in approximately 70% of the population, whereas IgG2 has no functional activity on the other common allelic form, FcγRIIa-R131, which contains arginine at position 131.11,61

**IgG SUBCLASSES: STRUCTURE AND PROPERTIES**

The molecular basis of IgG and FcγR interactions

The extracellular regions of the FcγR are structurally similar. Each low-affinity FcγR has two ectodomains, whereas the high-affinity FcγRI has a third domain but this is not directly involved in IgG binding.62

The interaction between the IgG subclasses and the FcγR is most comprehensively defined for human IgG1 by both X-ray crystallographic7,62,63 and mutagenesis structure/function analyses.64-66 These studies defined key regions of the IgG sequence required for interaction with their FcγRs.

Crystallographic analyses of the human IgG1-Fc complexed with FcγRI, FcγRII or FcγRIII show that these interactions are similar in topology, and asymmetric in nature. The second extracellular domain of the FcγR inserts between the two heavy chains. Here it makes contacts with the lower hinge of both H chains and with residues of the adjacent BC loop of one CH2 domain and the FG loop of the other. The N-linked glycan at asparagine 297 (N297) of the heavy chain is essential for the structural integrity of the IgG-Fc by affecting the spacing and conformation of the CH2 domains. Indeed, its removal ablates FcγR binding.67 Of particular relevance to therapeutic mAb development is that the normal low-affinity IgG interaction with FcγRIIIa is profoundly increased by the removal of the core fucose from the N297 Fc oligosaccharide.58

No crystallographic data are available for IgG2 or IgG4 Fc in complex with FcγR, but mutagenesis studies of the Fc and the FcγR revealed general similarity, but with critical differences, in the interaction of these subclasses with their cognate FcγR.

**Unique features of the IgG2 and IgG4 subclasses**

In IgG1, the stable interaction of the two heavy chains results from the combined effects of stable covalent inter-H-chain disulfide bonds and strong noncovalent interaction of the two CH3 domains (Table 3). In stark contrast, in IgG2 and IgG4 the interaction of the CH3 domains of each H-chain is weak. Residues 392, 397 and 409 (Eu numbering) profoundly affect the stability of these interactions. The difference at position 409 (R409 in IgG4 and K409 in IgG1) confers a 100-fold decrease in stability of the interface between the two CH3 domains of IgG4 compared with that of IgG1 (Table 3).69

Furthermore, the core hinge of IgG4 differs from IgG1 at position 228 (P228 in IgG1 and S228 in IgG4), resulting in unstable inter-heavy-chain disulfide bonds. This, together with the destabilizing amino acids in the CH3, confers the unique property of half-antibody (Fab arm) exchange between different IgG4 antibodies,69 thereby creating monovalent, bispecific IgG4 antibodies in vivo.69,70 The similarly unstable interactions between the CH3 domains in IgG2 are conferred by the interface residue M397; however, the stable inter-H-chain disulfide bonds of the core and upper hinge prevent half-molecule exchange (Table 3).69

In addition, IgG2 uniquely has three disulfide bond conformers (Table 3). The distinct conformers are formed when (1) each light chain is attached to the Cys315 residue of CH1 in the heavy chain (IgG2-A conformer), (2) both light chains attach to the upper hinge (IgG2-B) or (3) one light chain is attached to the CH1 Cys311 and one to the upper hinge of the other heavy chain (IgG2-AB).71 This results in distinct positioning of the Fabs relative to the Fc portions in the different conformers, which has implications for the interaction with antigen and the capacity of IgG2 to cross-link target molecules in the absence of FcγR binding, for example, in an agonistic mAb setting.72

It should also be noted that IgG3 has not been used in therapeutic mAbs despite its unique biology. The main impediment to its use are its physicochemical properties such as susceptibility to proteolysis and propensity to aggregate that present challenges to industry-scale production and stability but protein engineering is attempting to overcome these hurdles.73

**Therapeutic antibody design: improving mAb potency**

Many factors affecting FcγR-dependent responses in vivo come into play during mAb therapy. The
experience of three decades of clinical use of mAbs taken together with our extensive, albeit incomplete, knowledge of IgG and FcγR structure and immunobiology provides a war chest for the innovative development of new and highly potent mAbs through the manipulation of their interaction with the FcγR.

Therapeutic mAb engineering strategies are directed by many factors including the biology of the target, the nature of the antigen, the desired MOA and possibly the anatomical location of the therapeutic effect, and thus to optimize potency for a desired response, the context of use is critical.

The nature of the IgG isotype

Different capabilities for the recruitment and activation of the different immune effector functions are naturally found in the Fc regions of the human IgG subclasses. Thus, to achieve a desired MOA, the different IgG subclasses are important starting points for the selection and engineering of the optimal mAb Fc. IgG1 is, in many ways, a proinflammatory or “effector-active” subclass, as it can initiate the complement cascade and is a “universal” FcγR ligand. Notwithstanding it is also a ligand for the inhibitory FcγRIIb, IgG1 elicits proinflammatory responses through all activating-type FcγRs, including ADCC, ADCP and cytokine release.

Because of their more restricted FcγR-binding profile, IgG2 and IgG4 have offered some choice in potentially avoiding FcR effector function without the need for Fc engineering. They have been used as the backbone for therapeutic mAbs either because recruitment of patients’ effector functions was unlikely to be necessary for the primary MOA of the mAb or is possibly detrimental to the desired therapeutic effect. However, the use of these unmodified “inert” subclasses is not without consequences and underscores the need for Fc engineering to modify FcγR interactions—See the “Attenuating and ablating FcγR related functions of IgG” section.

Thus, the choice of IgG subclass for therapeutic mAb engineering is an important first step for engineering of novel mAbs of improved specificity, potency and safety.

Fc engineering for enhanced anticancer therapeutics

IgG1 is the predominant subclass used in the development of cytotoxic mAbs where induction of an activation-type response, ADCC or phagocytosis, is considered desirable. Cytotoxic mAb cancer therapeutics can control disease progression by one or more mechanisms. Their MOAs include direct induction of apoptotic cell death of the cancer cell (anti-CD20, anti-CD52) or blocking receptor signaling (anti-HER2, anti-EGFR). They may also harness FcγR effector functions, including ADCC in the tumor microenvironment. The approved mAbs, rituximab (anti-CD20), trastuzumab (anti-HER2) and cetuximab (anti-EGFR), are formatted on a human IgG1 backbone and all require activating-type FcγR engagement for optimal therapeutic activity. This presents an example where context of therapeutic use is critical for therapeutic mAb design. IgG1 antibodies bind both the activating FcγR (e.g. FcγRIIa) and the inhibitory FcγRIIB. In some environments effector cells will coexpress FcγRIIB together with FcγRI, FcγRIIa and FcγRII, as may occur on a tumor-infiltrating macrophage. Therapy with an IgG1 anti-cancer cell mAb may then be compromised by the inhibitory action of FcγRIIB upon the ITAM signaling of the activating FcγR as both types of receptor would be coengaged on such an effector cell by the mAb bound to the target cell. This leads to reduced therapeutic mAb potency. Thus, the relative contributions of the activating (A) and inhibitory (I) FcγR to the response by an effector cell, the A-to-I ratio, may be an important determinant in clinical outcome of therapeutic mAb activity, that is, the higher the A-to-I ratio, the greater the proinflammatory response induced by the therapeutic mAb or conversely the lower the A-to-I ratio, the greater the inhibition or dampening of the proinflammatory response.

Thus, the challenge for the development of more potent FcγR effector mAbs is to overcome three major obstacles. First, improving activation potency by selectively enhancing interaction with activating-type FcγR, particularly FcγRIIa owing to its predominant role in ADCC-mediated killing of tumor cells. Second, reducing binding interactions with the inhibitory FcγRIIB. These two approaches improve the FcγR A-to-I ratio of cytotoxic IgG1 mAbs. Third, overcoming the significant affinity difference in the interaction with the main FcγRIII allelic forms of FcγRIIIa-Y158 and FcγRIIIa-F15876,83,84 which appears to be an important source of patient variability in responses to therapeutic mAb treatment of cancer.

At the time of writing, some mAbs with improved potency are coming into clinical use. Their improved action has been achieved by modifying the N-linked glycan or the amino acid sequence of the heavy-chain Fc (Table 4).

Modification of the Fc glycan

The typical complex N-linked glycan attached to N297 of the heavy chain includes a core fucose. Antibodies that lack this fucose have approximately 50-fold improved binding to FcγRIIIa and FcγRIIB and importantly retain
Table 4. Fc or hinge-engineered monoclonal antibodies (mAbs) approved or in advanced clinical development.

| mAb name     | Target                                      | IgG backbone | Fc modification                                  | Effect on mAb                                      | Therapy area                                      | Most advanced development stage |
|--------------|---------------------------------------------|--------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------|---------------------------------|
| Andecaliximab| Matrix Metalloproteinase 9 (MMP9) 1          | IgG4          | S228P                                            | Stabilize core hinge                              | Oncology                                         | Phase III                      |
| Anifrolumab  | Interferon alpha/beta receptor 1            | IgG1          | L234F, L236E, P331S                              | Mimic IgG4 hinge and its CH2/F/G loop; plus ablate FcR binding | Immunology                                       | Phase III                      |
| Atezolizumab | PD-L1                                        | IgG1          | Aglycosylated (N297A)                            | Ablate FcR binding                                | Oncology                                         | Marked                          |
| Benralizumab | Interleukin 5                               | IgG1          | Afucosylated                                     | Selectively enhance FcRRIII interaction           | Respiratory dermatology; ear nose throat disorders; gastrointestinal; hematology; immunology; central nervous system; oncology | Marked                          |
| Durvalumab   | PD-L1                                        | IgG1          | L234F, L236E, P331S                              | Mimic IgG4 hinge and its CH2/F/G loop; plus ablate FcR binding | Oncology                                         | Marked                          |
| Evinacumab   | Angiopoietin-related protein 3              | IgG4          | S228P                                            | Stabilize core hinge                              | Metabolic disorders                              | Phase III                      |
| Inebilizumab | CD19                                         | IgG1          | Afucosylated                                     | Selectively enhance FcRRIII interaction           | Central nervous system; oncology                 | Phase III                      |
| Ixekizumab   | Interleukin 17A                             | IgG4          | S228P                                            | Stabilize core hinge                              | Dermatology; immunology; musculoskeletal disorders | Marked                          |
| Margetuximab | HER2                                         | IgG1          | F234L, L236V, R329P, Y300L, P319L                | Selectively enhance FcRRIII interaction           | Oncology                                         | Phase III                      |
| Mogamulizumab| C–C chemokine receptor type 4 (CCR4)        | IgG1          | Afucosylated                                     | Selectively enhance FcRRIII interaction           | Central nervous system; oncology                 | Marked                          |
| Tafasitamab  | (MOR208 XmAb 5574)                          | IgG1          | S238D, I332E                                     | Selectively enhance FcRRIII interaction           | Oncology                                         | Phase III                      |
| Nivolumab    | PD-1                                         | IgG4          | S228P                                            | FcRRIII interaction                               | Infectious disease; oncology                     | Marked                          |
| Obinutuzumab | CD20                                         | IgG1          | Afucosylated                                     | Selectively enhance FcRRIII interaction           | Immunology; oncology                             | Marked                          |
| Ocaratuzumab | CD20                                         | IgG1          | P247I, A389Q                                    | Selectively enhance FcRRIII interaction           | Oncology                                         | Phase III                      |
| Pembrolizumab| PD-1                                         | IgG4          | S228P                                            | Stabilize core hinge                              | Infection; oncology                              | Marked                          |
| Roledimab    | Rhesus D                                     | IgG1          | Afucosylated                                     | Selectively enhance FcRRIII interaction           | Hematological disorders                         | Phase III                      |
| Spesolimab   | (BI-655130)                                  | IgG1          | L234A, L236A                                    | Ablate FcR binding                                | Gastrointestinal; immunology                     | Phase III                      |
| Teplizumab   | CD3                                          | IgG1          | L234A, L235A                                    | Ablate FcR binding                                | Metabolic disorders                              | Phase II                       |
| Tislelizumab | PD-1                                         | IgG4          | S228P, E233P, F234V, L235A, L239A                | Stabilize core hinge; mimic IgG2 lower hinge for restricted | Oncology                                         | Phase III                      |

(Continued)
the weak, low-affinity binding to the inhibitory FcγRIIb. Furthermore, this glycoengineering increased binding affinity of the modified IgG1 mAb for both FcγRIIIa V138I and F158E allelotypes.\textsuperscript{86-88} Afucosyl versions of the tumor targeting mAbs such as anti-HER2, anti-EGFR and anti-CD20 had greater antitumor effects and increased survival,\textsuperscript{68,88,89} which is a reflection of the greatly increased, and selective, FcyRIII binding. Compared with their unmodified counterparts, the afucosyl mAbs showed dramatic improvement of FcγRIII-related effector responses such as stronger NK cell-mediated ADCC, or enhanced neutrophil-mediated phagocytosis through FcγRIIb and/or FcγRIIIa.\textsuperscript{23} However, certain neutrophil functions via FcγRIIa may be compromised.\textsuperscript{90,91}

There are six afucosylated antibodies in late-stage clinical trials or approved for treatment (Table 4). Notable is obinutuzumab, an afucosyl anti-CD20 mAb which nearly doubles progression-free survival in chronic lymphocytic leukemia patients as compared with the fucose-containing rituximab.\textsuperscript{68} This dramatic improvement in clinical utility reinforces the value of glycan engineering specifically and of Fc engineering generally in anticancer treatments.

**Mutation of the Fc amino acids**

Alteration of the amino acids in the heavy-chain Fc can alter IgG specificity and affinity for activating FcγRs. The anti-CD19 antibody MOR208 (XmAb 5574) is currently in phase III trials for the treatment of chronic lymphocytic leukemia.\textsuperscript{92} It contains two mutations in its IgG1 Fc, S329P and D357H, which increases affinity to FcγRIIIa, particularly the “lower-affinity” FcγRIIIa F158E allele. The mAb shows increased FcγRIII-mediated ADCC and phagocytosis in vitro, and reduced lymphoma growth in mouse models.

Margetuximab is an ADCC-enhanced IgG1 Fc-engineered variant of the approved anti-HER2 mAb trastuzumab in phase III for HER2-expressing cancers.\textsuperscript{56,93} Alteration of five amino acids (L235V, F243L, R292P, Y300L and P346L) enhanced binding to FcγRIIa which also had the additional effect of decreasing binding to the inhibitory FcγRIIb, and thereby increased its A-to-I FcγR ratio. This was apparent when compared with unmodified trastuzumab the margetuximab showed enhanced ADCC against HER2\textsuperscript{7} cells in vitro and demonstrated superior antitumor effects in an HER2-expressing tumor model in mice.

The anti-CD20 ocarituzumab is an Fc-engineered IgG1 mAb in late-stage clinical trials for the treatment of a range of cancers, including non-Hodgkin lymphoma and chronic lymphocytic leukemia.\textsuperscript{94} Two Fc mutations, F247I and A339Q, conferred about 20-fold increase in binding to both major allelic variants of FcγRIIIa and elicited sixfold greater ADCC than unmodified IgG1.

Thus, the engineering of the Fc domain or glycan for improved FcγRIIIa binding is a powerful tool to create more potent and clinically effective anticancer mAbs.

**Attenuating and ablating FcγR-related functions of IgG**

There are circumstances where binding to FcγR is unnecessary or undesirable in the MOA of a therapeutic mAb. Unmodified IgG irrespective of its subclass or intended therapeutic effect has the potential to engage an FcγR which may lead to suboptimal therapeutic performance or to unexpected and catastrophic consequences.\textsuperscript{57,59} Clearly reducing or eliminating FcγR interactions, when they are not required for therapeutic effect, may be desirable. Indeed, this had been addressed by the choice of IgG subclass or by modifying the Fc region. Indeed, most efforts in Fc engineering mAbs that have translated to an approved drug have focused on the reduction or elimination of FcγR interactions (Table 4).

One approach to minimize interactions with the activating FcγR has been the use of IgG4 or IgG2 backbones, which show a more restricted specificity for the activating FcγR and consequently have been traditionally, and simplistically, viewed as “inert” IgG subclasses. Notwithstanding the unexpected, and FcγR-dependent, severe adverse reaction induced by the IgG4 TGN1412 mAb, the IgG4 or IgG2
backbones have been successfully used in many settings. Indeed, checkpoint inhibitors, such as mAbs targeting CTLA-4 or the PD-L1/PD-1 interaction for the suppression of inhibitory signals that contribute to immune tolerance in the tumor microenvironment, are formatted on an IgG4 backbone. Pembrolizumab, nivolumab and cemiplimab are all anti-PD-1 antibodies currently used for cancer therapy and have been formatted on an IgG4 backbone. Similarly, the checkpoint inhibitor tremelimumab is an anti-CTLA-4 antibody formatted on an IgG2 backbone to avoid potential ADCC killing of target cells.

However, the use of IgG2 and IgG4 as “inert” subclasses is problematic. Both bind to the activating receptors FcγRIIa-H131 and FcγRI, respectively (Table 2), and initiate effector functions such as neutrophil activation and apoptosis induction. Interestingly, in experimental systems, cross-linking of anti-PD-1 IgG4-based mAb by FcγRI switched its activity from blocking to activatory. Moreover, IgG4 binds to FcγRIIb, which may scaffold the therapeutic mAb. Although scaffolding may be beneficial in some contexts, for example, in immune agonism, it can be disastrous and unexpected in others as it was for the anti-CD28 TGN1412 mAb. Thus, the IgG2 and IgG4 subclasses are not the optimum choice for “FcγR-inactive” mAbs, and so modifying the Fc is a more direct approach.

The complete removal of the heavy-chain N-linked glycan is well known to ablate all FcγR binding by dramatically altering the Fc conformation. Atezolizumab, an IgG1 anti-PD-L1 checkpoint inhibitor mAb, utilizes this strategy and eliminates FcγR and also complement activation.

Modification to the Fc amino acid sequence of the FcγR-contact regions can also be used to reduce FcγR binding. A widely used modification of IgG1 is the substitution of leucine 234 and 235 in the lower hinge sequence (L234V, L235S) with alanine (L234A, L235A). It is often referred to as the “LALA mutation” and effectively eliminates FcγR binding by more than 100 fold.65,105 and is used in teplizumab and spesolimab (Table 4).

A separate strategy has used combinations of amino acid residues from the FcγR-binding regions of IgG2 and IgG4, which have restricted FcγR specificity, together with other binding-inactivating mutations. The lower hinge amino acids of the IgG1 mAbs durvalumab (anti-PD-L1) and anifrolumab (anti-interferon-α receptor; Table 4) were modified to mimic the lower hinge of IgG4 (L234V, L235S). They additionally incorporated L234E in the lower hinge and F351S in the F/G loop of the CH2 domain to ablate FcγR binding by disrupting two major FcγR contact sites and also coincidently decreasing C1q activation.65

IgG4 mAbs have been similarly engineered to eliminate their interaction with FcγRI and FcγRIIb. The IgG4 anti-PD-1 antibody tislelizumab has had its FcγR contact residues in the lower hinge E233, L234, L235 substituted with the equivalent residues of IgG2 P, V, A (E233P, F234V, L235A) as well as the additional D265A mutation which disrupts a major FcγR contact in CH2. It also has substitutions in the core hinge S228P and the CH3 L309V and R409K to stabilize the H-chain disulfides and CH3 interactions, respectively, thereby preventing half- Ig exchange characteristic of natural IgG4. Collectively, these modifications create a stable IgG4 with no FcγR binding nor complement activation.

Thus, Fc engineering is an effective way to remove FcγR effector functions and may be preferable to using unmodified IgG2 or IgG4 backbones that have a more restricted repertoire of FcγR interactions but which are still able to induce certain effector functions.

Improving FcγRIIb interactions

Preferential or specific Fc engagement of FcγRIIb over the activating FcγR offers several potential therapeutic advantages for new mAbs in distinct therapeutic settings.

Improved recruitment of FcγRIIb immunoreceptor tyrosine inhibition motif-dependent inhibitory function

Harnessing the physiological inhibitory function of FcγRIIb by mAbs that target ITAM receptors has the potential to shut down ITAM-dependent signaling pathways of major importance in antibody pathologies. Such ITAM signaling receptors include the BCR complex on B cells which is active in systemic lupus erythematosus, the FcεRI on basophils and mast cell subsets in allergies or the activating-type FcγR on a variety of innate leukocytes in antibody-mediated tissue destruction. In such scenarios, the ITAM signaling receptor complex that is targeted by the therapeutic mAb must be co-expressed on the cell surface with the inhibitory FcγRIIb. This permits coengagement with ITAM signaling receptor by the Fab of the mAb and inhibitory FcγRIIb by its Fc which is the critical requirement in the inhibitory MOA for such therapeutic mAbs (Figure 1).

Obsexelimab (also known as XmAb5871; Table 4), currently in early clinical testing in inflammatory autoimmune disease, is an IgG1 mAb that targets CD19 of the BCR complex. It contains two Fc modifications, S267E and L328F (also known as “SELF” mutations), that selectively increased FcγRIIb binding by 400-fold to about 1 nM, which results in powerful suppression of BCR signaling and the proliferation of primary B cells.
The anti-IgE mAb omalizumab is an IgG1 mAb approved for the treatment of allergic disorders. Among similar but Fc-engineered IgG1 mAb XmAb7195, currently in early clinical testing, contains the affinity-enhancing SELF modifications. Both mAbs sterically neutralize the interaction between IgE and its high-affinity receptor FcεRI to prevent basophil and mast cell activation. However, XmAb7195 exhibited more efficient removal (sweeping; discussed later) of circulating IgE and also inhibited B-cell IgE production, presumably by binding to the IgE BCR on the B-cell surface and coclustering with FcγRIIb via its affinity-enhanced Fc domain. Thus, XmAb7195’s selective modulation of IgE production by IgE+ B cells in addition to its enhanced clearance of IgE may offer significantly improved therapeutic benefits in allergy therapy beyond simple IgE neutralization. The “SELF” mutations have also been used in agonistic mAbs (discussed later).

One cautionary note is that the arginine 131 (R131) of the IgG-binding site in FcγRIIb is critical for the enhanced affinity binding of “SELF”-mutated Fcs but it is also present in the activating-type “high responder” FcγRIIa-R131. Thus, antibodies modified with “SELF” have very-high-affinity binding to FcγRIIa-R131 with a potentially increased risk of FcγRIIa-dependent complications in patients expressing this allelic form, although, so far, none have been reported in clinical trials. However, an alternative set of six Fc mutations, termed “V12” (P238D, E233D, G237D, H268D, P271G and A330R), potently enhanced FcγRIIb interaction 200-fold, conferring the enhanced agonistic activity of anti-CD137 antibody and an anti-OX40 mAb.

**FUTURE ENGINEERING STRATEGIES**

Monoclonal antibodies are potent therapeutics in a number of chronic or once incurable diseases. However, there is still extensive unmet clinical need as well as considerable room for improvement in many existing therapeutics.

Further understanding of how antibody structure affects FcγR function is essential for future development of more potent and effective mAbs. Already, engineering of the IgG Fc and its glycan has proved a potent and effective approach for increasing the clinical effectiveness, functional specificity and safety of therapeutic mAbs and is an emerging pathway to the development of the “next-gen” therapeutics.

Future directions in the development and use of therapeutic antibodies should increasingly mimic normal protective antibody responses, which are polyclonal and elicited in the context of innate receptor engagement which includes the FcR as well as other powerfully responsive systems including the Toll-like receptors and complement receptors. Furthermore, the mixed subclass nature of these normal antibody responses suggests that circumstances may arise in therapeutic strategies where there is value in having distinctly modified Fcs for the nuanced engagement of different FcγR family members.
Treatments comprising multiple mAbs and immune stimulants are under investigation in infectious disease for neutralization coverage of variant strains. Indeed, such an approach may be most effective in emerging infectious disease such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The use of multiple mAbs tailored for distinct effector functions and targeting different epitopes will maximize the opportunity for cocktails of effector functions in different types of diseases. Indeed, in a small but contemporary example outside of infectious disease, the FDA-approved combination in adenocarcinoma therapy uses a cocktail of two mAbs, pertuzumab and trastuzumab, against Her2.123

Rather than one type of Fc to conquer all, the combined use of appropriately selected mAbs whose individual components are enhanced for the engagement of different FcγR members may utilize multiple components of the spectrum of effector responses on offer by the immune system. Such “next-gen” biologics will begin to realize the full potential of FcγR-mediated antibody immune therapeutics and offer transformational change for the treatment of intractable and incurable diseases.

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CONFLICT OF INTEREST
None.

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