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# Signaling by Antibodies: Recent Progress

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## Keywords

IgG, Fc receptors, effector function, immunomodulation, immunity, inflammation

## Abstract

IgG antibodies mediate a diversity of immune functions by coupling of antigen specificity through the Fab domain to signal transduction via Fc-Fc receptor interactions. Indeed, balanced IgG signaling through type I and type II Fc receptors is required for the control of proinflammatory, anti-inflammatory, and immunomodulatory processes. In this review, we discuss the mechanisms that govern IgG–Fc receptor interactions, highlighting the diversity of Fc receptor–mediated effector functions that regulate immunity and inflammation as well as determine susceptibility to infection and autoimmunity and responsiveness to antibody-based therapeutics and vaccines.

## INTRODUCTION

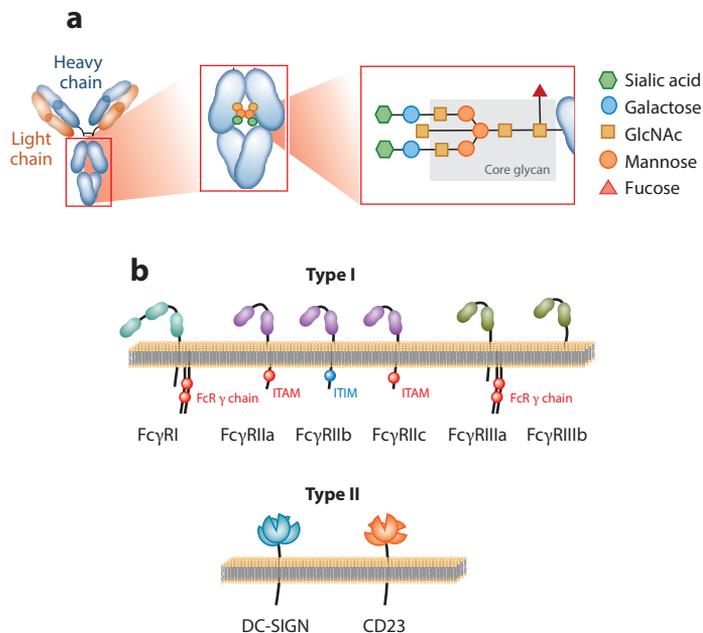
IgG is the primary antibody type secreted by memory B cells and plasma cells and is the dominant isotype involved in adaptive immune responses. The activity of IgG antibodies is determined by the Fab domain, which binds to antigen, and the Fc domain, which signals through distinct types of receptors (Fc $\gamma$  receptors; Fc $\gamma$ Rs) to mediate cellular effector functions that are required for in vivo protection against many infections and malignancies. In addition to directly mediating protective immune functions, IgG immune complexes signal through Fc $\gamma$ Rs to modulate adaptive immune responses, enhancing T cell activation and selection of high-affinity B cells. Here, we discuss IgG signaling pathways that are involved in mediating immunity and review recent studies demonstrating the critical role of signaling by antibodies in vivo.

## STRUCTURAL AND FUNCTIONAL HETEROGENEITY OF IgG

Because the majority of Fc $\gamma$ Rs exhibit low affinity for monomeric IgG, signaling by IgG molecules is mediated by multivalent IgG-antigen immune complexes that enable avidity-based Fc-Fc $\gamma$ R interactions and, in turn, Fc $\gamma$ R aggregation and signaling. The specific Fc $\gamma$ R engaged by a given Fc domain is dictated by Fc structure, which is determined by the IgG subclass and composition of an *N*-linked glycan present at Asn297 within the CH2 domain of the Fc (**Figure 1a**) (1, 2). On the basis of binding either an open or a closed conformation taken by the Fc domain, Fc $\gamma$ Rs can be distinguished as type I or II, respectively. These two receptor families transduce distinct effector and modulatory pathways; thus, it is the structure of the Fc domain that dictates IgG signaling.

IgG is composed of two identical heavy chains and two identical light chains, linked by interchain disulfide bridges. IgG subclasses are determined by the protein sequence of the heavy chains, with all subclasses pairing with either  $\kappa$  or  $\lambda$  light chains. A basic Y-shaped structure is common to all IgG subclasses and results from a hinge that separates the CH1 domain and light chains, comprising the Fab domain, from the CH2 and CH3 domains, which constitute the Fc domain (**Figure 1a**). While elaboration of a  $\kappa$  versus a  $\lambda$  light chain is not known to modulate IgG activity, the heavy chain subclass can have a profound impact on the in vivo activity of IgG antibodies, as it is one of the two determinants of Fc-Fc $\gamma$ R engagement and thus IgG-mediated signaling. Humans and mice produce four different IgG heavy chain subclasses, termed IgG1–4 in humans and IgG1, 2a (or 2c in some inbred strains), 2b, and 3 in mice. Protein homology between subclasses of human or mouse IgG is more than 90%, but differences that exist at the hinge-proximal CH2 domain, the interface of Fc-Fc $\gamma$ R interactions, confer distinct biological activities to IgG subclasses by determining Fc $\gamma$ R binding affinity. Each subclass is distinct in its ratio of binding to activating over inhibitory Fc $\gamma$ Rs (*A/I* ratio), with human IgG1 and IgG3 or mouse IgG2a/2c having the highest *A/I* ratios. In addition to distinct Fc $\gamma$ R binding affinities, the abundance of circulating IgG subclasses varies, with IgG1 in highest abundance in humans, followed by IgG2>IgG3>IgG4.

Composition of the biantennary *N*-linked glycan attached at Asn297 of human CH2 domains is the second determinant of Fc domain structure. A core heptasaccharide glycan is required to maintain the Fc domain in a conformational state that is permissive for type I or type II Fc $\gamma$ R binding. The core structure of this glycan can be modified by additional saccharide units that determine specific Fc-Fc $\gamma$ R interactions (**Figure 1a**). For example, sialylated glycoforms are thought to destabilize the quaternary structure of the Fc domain, conferring binding capacity of Fc to type II Fc $\gamma$ Rs (3). Glycan structures lacking a core fucose residue enhance affinity for the activating type I Fc $\gamma$ RIIIa through stabilized glycan-glycan interactions between the Fc domain and Fc $\gamma$ RIIIa (4). Fc glycan modifications are dynamic, with major changes reported to occur during pregnancy (5), in some malignancies (6), in response to vaccination (7), and during viral infection in select



**Figure 1**

Overview of the structural characteristics of (a) IgG and (b) FcγRs. IgG antibodies comprise two identical heavy and light chains linked by interchain disulfide bonds. At the core of the Fc domain, an *N*-linked glycan structure is conjugated at a highly conserved site at the CH2 domain (Asn297 for human IgG1), which regulates the conformation of the Fc domain and determines binding to type I and type II FcγRs. The structure and composition of the Fc-associated glycan can be dynamically regulated through the addition of specific sugar moieties to the core glycan structure (*grey shaded area*), which in turn determines the binding affinity of the Fc domain for type I and type II FcγRs. Despite their common property of interacting with the IgG Fc, type I and type II FcγRs present distinct structural and functional differences and have differential capacity to induce diverse immunomodulatory consequences that affect several aspects of innate and adaptive immunity. Abbreviations: ITAM, immunoreceptor tyrosine activation motif; ITIM, immunoreceptor tyrosine inhibition motif.

individuals (T.T. Wang & J.V. Ravetch, unpublished observation). Specific inflammatory diseases are also associated with Fc glycan modifications, such as diminished galactosylation and sialylation on disease-associated IgG specificities in rheumatoid arthritis (8) and in Wegener granulomatosis (9).

### Type I and Type II Fcγ Receptors

Type I FcγRs belong to the immunoglobulin superfamily and share many structural and functional properties (**Figure 1b**). The extracellular domain of type I FcγRs comprises either two or three immunoglobulin domains and interacts with the hinge-proximal region of the CH2 domain of IgG in a 1:1 stoichiometric complex. Type I FcγRs share highly conserved intracellular motifs that mediate signaling upon receptor cross-linking by IgG immune complexes, yet distinct functional outcomes of signaling define each receptor. Type I FcγRs can be classified as activating or inhibitory based on the presence of intracellular activating (immunoreceptor tyrosine activation motif; ITAM) or inhibitory (immunoreceptor tyrosine inhibition motif; ITIM) signaling motifs. Three activating FcγRs are expressed in mice (FcγRI, FcγRIII, and FcγRIV) and three in humans (FcγRI, FcγRIIIa, and FcγRIIIb); FcγRIIb is the single inhibitory FcγR expressed in both species.

**Table 1** Expression patterns<sup>a</sup> of type I and type II FcγRs among human leukocyte types

Cell type	Type I						Type II	
	FcγRI	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb	DC-SIGN	CD23
B cells	–	–	+	–	–	–	–	+
T cells	–	–	–	–	–	–	–	I
NK cells	–	–	–G	+G	+	–	–	–
Monocytes	+	+	+	–	+/-	–	+/-	I
Neutrophils	I	+	+	–	–	+	–	I
Eosinophils	I	+	+	–	–	I	–	I
Macrophages	+/-	+	+	–	+/-	–	+/-	I
Dendritic cells	I	+	+	–	I	–	+	–

<sup>a</sup>Expression patterns represented as follows: +, constitutive; –, none; G, depends on FcγR genetic variation; I, inducible; +/- restricted to certain subsets.

Each FcγR has a distinct pattern of cellular expression (**Table 1**), with engagement of activating FcγRs inducing positive mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and proinflammatory cytokine production. Signaling through the inhibitory receptor, FcγRIIb, results in negative functions such as inhibition of inflammatory immune responses. FcγRI is the only high-affinity FcγR, capable of binding monomeric IgG ( $K_D = 10^{-9}$ – $10^{-10}$  M for human IgG1); all other type I FcγRs exhibit lower affinity for IgGs, with precise affinities varying by subclass ( $K_D$  for human IgG1 =  $10^{-5}$ – $10^{-7}$  M). Whether Fc domains within IgG immune complexes engage activating or inhibitory type I FcγRs is determined by the IgG subclass and the Fc glycan composition, as described above (1).

Type II FcγRs are C-type lectins that have been studied in various biological contexts for some time, but their identification as receptors for IgG antibodies occurred relatively recently (3, 10). Type II FcγRs with known functional activities include DC-SIGN (mouse ortholog SIGN-R1) and CD23, which share an oligomeric structure that is stabilized through an  $\alpha$ -helical coiled-coil stalk motif at the extracellular receptor domain. The primary determinant of IgG binding to type II FcγRs is the presence of sialylated Fc glycoforms, which destabilize the Fc structure, enabling assumption of a conformation that is the ligand for type II FcγRs. As with the majority of type I FcγRs, type II FcγRs are low affinity for their Fc ligand ( $K_D$  for hIgG1 =  $10^{-5}$ – $10^{-7}$  M) and they have distinct immunomodulatory activities: DC-SIGN signaling is involved in the anti-inflammatory activity of IgG through the production of cytokines such as IL-33 that are involved in setting thresholds for inflammatory myeloid cell activation (11), whereas CD23 mediates selection of high-affinity B cells during maturation of antibody responses (7); both receptors may be involved in additional modulatory functions that have yet to be characterized. Specific type I and type II FcγR signaling pathways and downstream functions are described throughout this review.

### Species Specificity in IgG Signaling

All known animal models, including nonhuman primates, express species-specific IgG subclasses and FcγRs that are unique in structure, function and cellular distribution. Such interspecies variation limits interpretation of data from mixed-species systems, i.e., in experiments using human IgGs in nonhuman primates or in mice. While affinities between human IgGs and FcγRs from other species can be determined and may guide interpretation of mixed-species systems, use of data from these systems to reliably inform development of antibody therapeutics is, at this time, nearly

impossible. Models with IgGs and FcγRs that are species matched can be easier to interpret when the animal system has been well defined, as with C57BL/6 and BALB/c mice. Still, major hurdles exist. One example of discord that can prevent extrapolation of *in vivo* mouse data is the difference in receptor expression on human monocyte- and mouse monocyte-derived dendritic cells. For example, human monocyte-derived dendritic cells express one activating type I receptor, FcγRIIa, along with the inhibitory FcγRIIb, whereas mouse monocyte-derived dendritic cells express all murine type I FcγRs. Another example from nonhuman primates is found in neutrophil FcγR expression: while human neutrophils express FcγRIIa, FcγRIIb, and FcγRIIIb, neutrophils from nonhuman primate species like rhesus macaques do not express FcγRIIIb but do express FcγRI in addition to FcγRIIa and FcγRIIb. These differences in receptor expression can result in major differences in cellular functions—one example is mouse ADCC, which is mediated primarily by mouse IgG2a engagement of murine FcγRIV on macrophages; mouse NK cells do not express FcγRIV and thus do not significantly contribute to antibody-mediated cellular depletion *in vivo* by IgG2a (12). In contrast, ADCC in humans is mediated through the human IgG1 subclass engaging FcγRIIIa, which is expressed by both macrophages and NK cells; both cell types have been suggested to contribute to ADCC in humans and are influenced by the allele of FcγRIIIa expressed and the fucosylation state of the IgG1 Fc. The best model currently available for the *in vivo* study of human FcγRs is a mouse model in which endogenous mouse FcγR genes were deleted and human FcγRs are instead expressed under the control of their endogenous human promoters. This FcγR humanized mouse strain recapitulates the human type I FcγRs in form, function, and cellular expression (13).

## INTERPLAY BETWEEN TYPE I AND TYPE II FcγRs IN REGULATING IMMUNE RESPONSES

Interactions between FcγRs and the Fc domain of IgG antibodies activate signaling pathways that vary depending on the FcγR and cell type that is engaged; each Fc-FcγR interaction has the potential to modulate a diverse set of immunoregulatory processes (**Table 2**) (1). As discussed in the previous section, the affinity of IgG for different FcγRs is regulated by the Fc domain structure, which is determined by the primary amino acid sequence of the Fc domain (IgG subclass) and the associated *N*-linked glycan. While Fc structure regulates Fc-FcγR interactions and subsequent signaling events, several factors influence the capacity of FcγR-mediated signaling to modulate immune processes. For example, each FcγR exhibits a distinct expression pattern among leukocyte populations, with different cell subsets commonly coexpressing more than one FcγR type (**Table 1**) (1, 13). Expression levels and, to a degree, the pattern of FcγR expression can fluctuate during leukocyte differentiation and activation, with each cell type generally coexpressing more than one FcγR type at any time (**Table 1**). Additionally, several FcγR genes can be induced acutely by chemokines and/or cytokines present at sites of inflammation, infection, or tissue injury, thereby influencing FcγR-mediated expression and, in turn, effector activities (11, 14, 15). The specific FcγR expression pattern on a cell determines whether receptor cross-linking will lead to cellular activation, ADCC and phagocytosis, release of cytokines and chemokines, leukocyte differentiation and survival, and/or modulation of T cell and B cell responses (16). In this section, we focus on the key role of IgG antibodies in modulating immune signaling through selective engagement of different FcγRs (**Table 2**).

### Cytotoxicity of IgG-Coated Targets

All cytotoxic effector functions induced upon FcγR-IgG interactions are mediated through activating type I FcγRs. One of the first activities described for FcγRs was their capacity to mediate

**Table 2 Overview of FcγR-mediated effector functions in different leukocyte types**

	Cell type	Role of FcγR <sup>a</sup>		Short-term effects	Long-term effects
		+	–		
Myeloid	Granulocytes	FcγRIIa, FcγRIIIb <sup>b</sup>	FcγRIIb	Degranulation, ROI production, phagocytosis	Release of proinflammatory molecules, cell survival and motility, impact on other myeloid leukocytes
	Monocytes	FcγRIIa, FcγRIIIa <sup>c</sup>	FcγRIIb	Phagocytosis, cytokine and chemokine expression	Monocyte recruitment and differentiation, cell survival, stimulation of proinflammatory pathways
	Macrophages	FcγRIIa, FcγRIIIa, DC-SIGN	FcγRIIb	Phagocytosis, cytokine and chemokine expression	Influence on macrophage polarization and responsiveness to IgG-mediated inflammation
	Platelets	FcγRIIa		Degranulation, platelet activation	Platelet binding to leukocytes, impact on leukocyte functional activity
	Dendritic cells	FcγRIIa	FcγRIIb	Cell maturation, upregulation of costimulatory molecules	Enhanced antigen processing and presentation, impact on T cell responses
Lymphoid	B cells	CD23, FcγRIIb		B cell selection	Generation of high-affinity IgG responses
	Plasma cells		FcγRIIb	B cell survival	Regulation of plasma cell apoptosis, control of IgG production
	NK cells	FcγRIIIa		Cell activation, degranulation	Cytotoxicity, cell survival, impact on other effector leukocytes, generation of immune complexes

<sup>a</sup>Either (+) activates or (–) inhibits downstream effects.

<sup>b</sup>Function unknown; possibly activating.

<sup>c</sup>Expressed on certain subsets.

clearance or cytotoxicity of IgG-coated targets, ranging from small antigens (toxins, infectious pathogens, etc.) to whole cells. Cross-linking of activating type I FcγRs by IgG immune complexes triggers a cascade of signaling events that culminate in the elimination of the IgG-opsonized target. Following receptor clustering and oligomerization by IgG immune complexes, phosphorylation of receptor ITAM domains occurs, promoting the recruitment and activation of several tyrosine kinases of the Src and Syk families. This leads to activation of the phosphatidylinositol-3-kinase (PI3K)–protein kinase C (PKC) pathway and to Ca<sup>2+</sup> mobilization and consequently cellular activation (17, 18). In addition to these early signaling events, several other late signaling pathways are activated, including the MEK and MAP family kinases and the Ras pathway, which in turn induce the transcription of proinflammatory cytokines and chemokines with divergent effects on cellular survival and differentiation (19).

Although intracellular signals transduced upon receptor cross-linking are common to all leukocytes expressing activating type I FcγRs, the precise biological responses following FcγR engagement vary greatly between different leukocyte types. For example, granulocytes mediate biological effects within minutes following activation of FcγR-mediated pathways by IgG immune complexes (20). Granulocyte effector functions aim to destroy invading pathogens, often by releasing microbicidal molecules that either are preformed and stored in specialized cytoplasmic granules or are generated, de novo, upon FcγR engagement. For example, FcγR cross-linking leading to

the activation of Syk and Src family kinases triggers the production of reactive oxygen intermediates (ROIs) through formation and activation of the NADPH-dependent oxidase complex (21, 22). Additionally, Fc $\gamma$ R cross-linking induces activation of the PKC pathway and the subsequent increase in intracellular Ca<sup>2+</sup> stimulates mobilization and release of preformed cytotoxic or proinflammatory molecules (20). These molecules vary among granulocyte subtypes but generally include serine proteases (elastase, cathepsins, and collagenases) and other enzymes (peroxidase, alkaline phosphatase), antimicrobial peptides and proteins (lysozyme, defensins, lactoferrin), and lipid mediators (leukotrienes) with potent proinflammatory activity (23–25). In addition, binding of multimeric IgG immune complexes to Fc $\gamma$ Rs triggers receptor cross-linking and oligomerization, which induces receptor internalization and activation of downstream signaling pathways that facilitate actin remodeling, endosomal uptake, and sorting (26, 27). Indeed, granulocytes are among the most potent phagocytic leukocytes and can efficiently engulf and eliminate IgG-opsonized bacteria, fungi, and other cellular antigens (28). These effects along with the production and release of ROIs and cytotoxic proteolytic compounds represent the two main strategies that granulocytes employ to achieve cytotoxicity of IgG-coated targets.

Similar signaling processes are also initiated upon engagement of the activating Fc $\gamma$ RIIIa on NK cells, which triggers cellular activation and degranulation. Analogous to granulocyte activation and degranulation following Fc $\gamma$ R cross-linking, engagement of Fc $\gamma$ RIIIa on NK cells is associated with ITAM phosphorylation and rapid increase in the intracellular Ca<sup>2+</sup> levels, which triggers the release of granular contents, including perforin and granzymes (29, 30). The release of these potent enzymes in close proximity to IgG-coated targets induces formation of pores at the target cell membrane and stimulation of proapoptotic pathways, ultimately leading to cell death.

Elimination of IgG-opsonized targets by macrophages, in contrast to granulocytes and NK cells, is achieved primarily through phagocytic processes, and not through the immediate release of cytotoxic and cytolytic compounds. Indeed, several studies have shown that Fc $\gamma$ R-expressing macrophages mediate cytotoxic activity *in vivo* through phagocytic clearance of IgG-coated cells. In seminal experiments using IgG-opsonized erythrocytes, their uptake was observed to be mediated exclusively by splenic macrophages in an Fc $\gamma$ R-dependent process (31, 32). Similarly, in mice humanized for Fc $\gamma$ Rs, specific depletion of macrophages by clodronate liposomes completely abrogated the cytotoxic activity of antibodies against various surface antigens, further confirming the central role of macrophages in IgG-mediated cytotoxicity *in vivo* (12).

With the exception of NK cells that only express Fc $\gamma$ RIIIa, activating type I Fc $\gamma$ R signaling is always balanced on effector leukocytes by signaling through coexpressed inhibitory Fc $\gamma$ RIIb; thus, Fc $\gamma$ R-mediated effector functions are the result of cumulative signaling from activating and inhibitory type I Fc $\gamma$ Rs (2). For example, the *in vivo* activity of cytotoxic therapeutic antibodies depends on a signaling balance that is weighted toward activating Fc $\gamma$ Rs (2, 12, 33–36). This was demonstrated in early experiments utilizing a monoclonal antibody (TA99) against a melanosome protein, gp75, in which it was evident that tumor clearance required activating Fc $\gamma$ R engagement, and IgG subclass or glycovariants of TA99 with enhanced capacity to engage activating Fc $\gamma$ Rs exhibited improved *in vivo* activity in a mouse model of metastatic melanoma (2).

Additional evidence on the importance of the balanced activity of activating and inhibitory Fc $\gamma$ Rs in regulating IgG-mediated inflammation comes from genetic association studies of Fc $\gamma$ R genetic variants. For several autoimmune and chronic inflammatory pathologies, disease susceptibility and/or severity is associated with genetic variants of Fc $\gamma$ R-encoding genes in the form of single-nucleotide polymorphisms or copy number variants that influence receptor expression, activity, or IgG binding affinity (37). For example, patients with systemic lupus erythematosus (SLE) are enriched for the low-binding alleles for Fc $\gamma$ RIIa, Fc $\gamma$ RIIIa, and Fc $\gamma$ RIIIb and have a

lower copy number of the *FCGR3B* gene, which is characterized by reduced FcγRIIIb expression levels (37–40). These variants exhibit reduced clearance of IgG immune complexes, which likely increases tissue immune complex deposition and inflammation. In addition, low copy number of *FCGR3B* is associated with the induction of ectopic expression of the inhibitory FcγRIIb in NK cells, likely reducing cytotoxic responsiveness (40). In contrast, high-affinity variants of FcγRIIIa (V158) and FcγRIIIb (NA1) are commonly associated with inflammatory diseases, such as autoantibody-induced vasculitis, idiopathic thrombocytopenia purpura, and rheumatoid arthritis (37). These cumulative studies demonstrate the key role of FcγR-IgG interactions in mediating cytotoxic effector responses and highlight that balanced activating and inhibitory FcγR signaling is required for optimal effector activity without pathogenic immune activation.

### Antigen Processing and Modulation of Antigen-Presenting Cell Function

Despite structural and functional differences, the different type I FcγRs follow an identical pattern of receptor cross-linking and oligomerization that ultimately leads to sequestration of IgG-FcγR complexes in intracellular vesicles for lysosomal degradation and antigen processing (26). Indeed, all type I FcγRs (with the exception of the FcγRIIb1 splice variant) have the capacity to mediate phagocytosis of IgG-opsonized targets (41). The efficiency of FcγR-mediated phagocytosis varies greatly between leukocyte types and is dependent on differentiation stage, cytoskeletal motility, cellular size, and expression levels of type I FcγRs and other receptors such as pattern-recognition receptors. Although both activating and inhibitory type I FcγRs can mediate phagocytosis in myeloid phagocytes, internalization of IgG-opsonized targets through activating FcγRs mediates more potent effector responses (42–44). For example, phosphorylation of ITAM domains after FcγR cross-linking leads to proinflammatory signaling that mediates cellular activation and proinflammatory cytokine and chemokine expression, which modulates leukocyte differentiation, functional activity, and survival. Uptake of IgG-coated targets through activating FcγRs is associated with enhanced endosomal maturation and lysosomal fusion, leading to more efficient antigen processing and presentation on MHC class II molecules (42, 45). Antigen processing and presentation resulting from FcγR-mediated internalization of immune complexes result in enhanced T cell activation (36, 46–48).

Apart from the direct effects on antigen uptake, processing, and presentation to MHC class II molecules, proinflammatory signals following type I FcγR engagement by IgG immune complexes also influence several aspects of the activity and function of antigen-presenting cells (APCs). For example, signaling through FcγRs can influence macrophage differentiation and polarization, as well as dendritic cell maturation and activity, which can affect T cell activation and tolerance. In steady-state conditions, human monocytic dendritic cells express two type I FcγRs: the inhibitory FcγRIIb and the activating FcγRIIa (1). Since these receptors are coexpressed on the surface of dendritic cells, proinflammatory signals from FcγRIIa are antagonized by the inhibitory signaling activity of FcγRIIb. This balance between activating and inhibitory FcγRs represents a key regulatory process of dendritic cell function, necessary to prevent uncontrolled dendritic cell maturation and maintain peripheral tolerance (49). Indeed, the expression levels of these two FcγRs are tightly regulated under homeostatic conditions, whereas certain mediators present at the inflammatory milieu can influence the expression of FcγRIIb or induce expression of additional activating type I FcγRs, thereby altering the threshold for IgG-mediated dendritic cell maturation. For example, IFN-γ induces the expression of the activating type I FcγR, FcγRI, and downregulates FcγRIIb expression. In contrast, IL-4 stimulates the expression of the inhibitory FcγRIIb (11, 14, 15, 48, 50). This fine-tuned interplay between activating and inhibitory FcγR expression determines the threshold for FcγR-mediated dendritic cell maturation and responsiveness to other stimuli [e.g.,

Toll-like receptor (TLR) ligands]. In the absence of costimulatory signals, stimulation of dendritic cells with IgG immune complexes often fails to induce robust cell maturation. Activation of proinflammatory signaling pathways through innate immune receptors is generally required to overcome the inhibitory activity of FcγRIIb and induce dendritic cell activation (12, 36, 46, 48, 50).

Skewing the balance of activating and inhibitory FcγR signaling has profound consequences for the maturation of APCs and subsequent generation of T cell responses. For example, preferential engagement of dendritic cell FcγRIIA through Fc domain engineering of anti-CD20 antibodies resulted in improved antigen-specific T cell responses in a model of CD20<sup>+</sup> lymphoma (12). Likewise, blocking FcγRIIb ligand binding activity using function-blocking antibodies or genetic deletion of the *Fcgr2b* gene greatly augments IgG immune complex-mediated cell maturation, resulting in upregulation of costimulatory molecules as well as enhanced antigen presentation and T cell activation (36, 48, 50). These studies further highlight the critical balance in signaling between different types of FcγRs that must be achieved to regulate dendritic cell activity associated with generation of robust adaptive immune responses.

As with dendritic cells, FcγR-mediated signaling can also influence the differentiation status and function of macrophages. Human macrophages exist as a continuum of diverse differentiation states with distinct functional and phenotypic characteristics (51). Although macrophage polarization was originally classified into two broad phenotypes—M1 and M2, which are induced by Th1 (IFN-γ) and Th2 (IL-4) cytokines, respectively—stimulation through FcγR-mediated pathways represents a distinct determinant for macrophage polarization, resulting in the induction of a characteristic activation phenotype (51). Engagement of activating type I FcγRs and stimulation of downstream signaling pathways in macrophages are associated with induction of proinflammatory cytokine and chemokine expression that alters their differentiation, function, and survival (16). However, when activating FcγR signals are coupled with TLR signaling, like TLR4 in nonpolarized macrophages, this synergistic signaling activity triggers induction of a specific polarization state that resembles the M2 phenotype (52, 53). This phenotype, also termed regulatory or M2b, is characterized by production of IL-10, IL-1, and IL-6 as well as by increased migratory and phagocytic capacity (52, 53). Apart from type I FcγRs, signaling through type II FcγRs on macrophages can influence their functional activity and responsiveness. For example, studies designed to identify the mechanistic basis of the anti-inflammatory activity of high-dose intravenous immunoglobulin (IVIG) treatment showed that engagement of the type II FcγR, DC-SIGN, by sialylated Fc domains of IgG antibodies triggers the expression of IL-33 by regulatory macrophages. This secreted IL-33, a potent Th2-polarizing cytokine, stimulates production and release of IL-4 by basophils, which, in turn, upregulates FcγRIIb on effector macrophages (11). Increased FcγRIIb expression alters the balance between activating and inhibitory type I FcγR signaling, raising the threshold for IgG-mediated inflammation in macrophages.

In addition, several studies utilizing mouse strains with genetic deletion of the different types of FcγRs provide evidence for the contribution of FcγR-mediated signaling to macrophage function and in vivo activity. For example, mice lacking activating type I FcγRs are nonresponsive to IgG-mediated inflammation (32, 52), whereas FcγRIIb<sup>-/-</sup> mice exhibit lower macrophage activation threshold upon challenge with immune complexes and present a more severe phenotype in models of immune complex-induced anaphylaxis, arthritis, and alveolitis (32, 52, 54). Furthermore, in mouse strains deficient for either FcγRIIb or SIGNR1, the mouse ortholog for human DC-SIGN, administration of high-dose IVIG or sialylated Fcs fails to provide anti-inflammatory activity in models of IgG-mediated inflammation (10, 11). These studies clearly highlight the importance of type I and type II FcγR signaling in the regulation of the activity and differentiation of APCs, with diverse consequences for immunity and inflammation (**Table 2**).

## B Cell Selection and Regulation of IgG Production

In the majority of leukocyte populations, expression of activating Fc $\gamma$ R<sub>s</sub> is coupled to expression of the inhibitory Fc $\gamma$ RIIb. In contrast, B cells constitutively express Fc $\gamma$ RIIb throughout their development, without coexpressing any activating type I Fc $\gamma$ R<sub>s</sub>. Fc $\gamma$ RIIb on B cells functions to balance the activating signals triggered by binding of antigen to the B cell receptor (BCR). In the absence of BCR signaling, exclusive engagement of B cell Fc $\gamma$ RIIb induces proapoptotic signals, whereas Fc $\gamma$ RIIb–BCR coengagement attenuates these apoptotic signals, leading to B cell survival (55, 56). This regulatory mechanism controls B cell selection, as B cells with low or no affinity for the antigen are eliminated through apoptosis, whereas those with high-affinity receptors receive prosurvival signals (56). Transition of B cells to plasma cells is associated with loss of BCR expression. In the absence of opposing BCR signaling, Fc $\gamma$ RIIb engagement by IgG immune complexes leads to plasma cell apoptosis; this negative feedback mechanism regulates plasma cell survival to prevent uncontrolled IgG production (56–58).

As described above, proapoptotic signaling activated through engagement of B cell Fc $\gamma$ RIIb alone is attenuated by coaggregation with BCR. At the same time, thresholds for B cell activation through BCR are determined by *cis* signaling through Fc $\gamma$ RIIb, which can occur when antigen within an IgG immune complex binds BCR and Fc domains within the same complex coengage Fc $\gamma$ RIIb. Immune complex engagement of Fc $\gamma$ RIIb leads to phosphorylation of the ITIM domains of Fc $\gamma$ RIIb, which, in turn, recruit SHIP and SHP2 phosphatases (55, 56, 58). These phosphatases directly interfere with BCR signaling by hydrolyzing phosphoinositide intermediates, such as phosphatidylinositol-3,4,5-triphosphate, thus preventing recruitment and activation of PH domain–containing kinases, such as PLC $\gamma$  and BTK and blocking induction of downstream signaling pathways (55, 56, 58). Studies using Fc $\gamma$ RIIb-deficient mice have revealed how critical the contribution of Fc $\gamma$ RIIb signaling is in setting the activation threshold for B cells, enabling high-affinity antibody responses and maintaining peripheral tolerance. Indeed, in the absence of Fc $\gamma$ RIIb, antibody responses are characterized by high titer and low affinity due to absence of selection of B cells based on affinity of BCR (49, 59). In addition, genetic variants of *FCGR2B* that influence receptor activity or expression are associated with increased susceptibility to autoimmunity. For example, promoter haplotypes of the *FCGR2B* gene that decrease Fc $\gamma$ RIIb expression levels are associated with SLE susceptibility and likely contribute to disease pathogenesis (60, 61). Additionally, several studies have reported an association between a single-nucleotide polymorphism (I232T) at the transmembrane region of Fc $\gamma$ RIIb and SLE (62). This polymorphism affects receptor partitioning to lipid raft membrane microdomains, thus diminishing Fc $\gamma$ RIIb signaling activity. This central role of Fc $\gamma$ RIIb in regulating BCR signaling activity represents a key determinant in the generation of antibody responses and B cell selection and affinity maturation, as well as maintenance of peripheral tolerance (49, 55, 56, 59).

Apart from the type I Fc $\gamma$ RIIb, B cells express the type II Fc $\gamma$ R CD23, which also participates in the regulation of B cell activity and generation of high-affinity antibody responses. Early studies on the role of CD23 showed that CD23 regulates IgE production and mediates the capture of IgE-containing immune complexes and transfer to CD11c<sup>+</sup> APCs, leading to enhanced CD4<sup>+</sup> T cell immunity (63). As CD23 also has the capacity to interact with sialylated IgG Fcs, more recent studies have demonstrated that IgG–CD23 interactions can also modulate B cell function. For example, studies have shown that IgG immune complexes containing sialylated Fc domains can drive the development of high-affinity IgG responses (3, 7). A recent study identified the mechanisms by which CD23 influences affinity of IgG responses; engagement of CD23 by sialylated IgG immune complexes was shown to upregulate B cell expression of Fc $\gamma$ RIIb, thus elevating the threshold for B cell selection, resulting in high-affinity IgG responses. This mechanism was

tested using a model of influenza hemagglutinin (HA) immunization in which it was shown that vaccination with sialylated Fc anti-HA immune complexes induced higher-affinity anti-HA IgG responses with increased breadth of *in vivo* protective activity. This effect was lost in CD23<sup>-/-</sup> mice or when wild-type mice were vaccinated with asialylated anti-HA immune complexes (7). These studies highlight the synergistic activity of type I and type II FcγRs to shape humoral immune responses by directly regulating B cell selection, IgG affinity, and plasma cell survival.

In the next sections, using specific examples of antibodies against neoplastic or infectious diseases, we review the role and function of type I and type II FcγRs in the *in vivo* protective activities of these antibodies. Numerous monoclonal antibodies with antitumor or antimicrobial activity are currently in development or already in clinical use, representing the promise of therapeutic modalities for several infectious diseases and tumors. Unraveling the complexity of Fc-FcγR interactions and the requirements for activation of specific FcγR-mediated pathways by these therapeutic monoclonal antibodies to achieve full protective activity has led to the development of Fc-optimized IgG therapeutics with improved clinical efficacy.

## SIGNALING AND FUNCTION OF ANTITUMOR ANTIBODIES

IgG antibodies are now routinely used for the treatment of a variety of human malignancies. The relative success of rituximab, the first monoclonal antibody approved by the US Food and Drug Administration (FDA) to treat cancer, led to the evaluation of numerous other IgG cancer therapeutics, resulting in many monoclonal antibodies being brought to the clinic over the last two decades. Cancer-targeting therapeutic antibodies can be mechanistically distinguished by the type of cell targeted by their Fab domains. They target either the tumor cells to directly mediate cytotoxicity or immune cells to potentiate antitumor immunity. It is now clear that, independent of the cell type being targeted by the Fab domain, the Fc domain of antitumor therapeutic antibodies must be optimized to engage the appropriate class of FcγRs on effector cells to achieve potent clinical activity. Therefore, the IgG isotype and specific Fc scaffold of these antibodies are critical for mediating full therapeutic efficacy.

### Cytotoxic Antitumor Antibodies

The development of hybridoma technology along with identification of antigens specific to or overexpressed on tumor cells, but not normal cells, enabled the exploitation of the fine specificity of antibodies to precisely target tumor cells. The first monoclonal antibodies that emerged as cancer therapeutics targeting tumor-associated antigens were rituximab, an anti-CD20 monoclonal antibody that targets malignant B cells in non-Hodgkin lymphoma and chronic lymphocytic leukemia; trastuzumab, an antihuman epidermal growth receptor 2 (HER2)/neu monoclonal antibody that targets HER2<sup>+</sup> tumor cells in breast cancers; and cetuximab, an anti-epidermal growth factor receptor (EGFR) monoclonal antibody for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer, and head and neck cancer. Multiple mechanisms of action were proposed for these antibodies: Fab-mediated pathways, such as the induction of cell death signaling pathways (rituximab) or receptor downregulating and blocking (trastuzumab and cetuximab), as well as Fc-mediated pathways, such as complement-dependent cytotoxicity or ADCC, were proposed based primarily on *in vitro* studies in a variety of experimental systems. Among these mechanisms, only engagement of activating type I FcγRs for depletion of target cells was found to be an absolute requirement for the *in vivo* activity of tumor-targeting monoclonal antibodies, including rituximab, trastuzumab, and cetuximab.

The most striking evidence for FcγR-mediated mechanisms determining the antitumor activity of cytotoxic antibodies *in vivo* comes from work showing that antitumor monoclonal antibodies

can be therapeutically potent in wild-type mice, but not in mice lacking the FcR  $\gamma$  chain, which is required for the expression and signaling of all murine activating Fc $\gamma$ Rs (64). Moreover, the antitumor effect of monoclonal antibody therapy was enhanced in mice lacking the inhibitory Fc $\gamma$ RIIb (47), implicating the signaling balance between activating and inhibitory Fc $\gamma$ Rs in determining the potency of antitumor cytotoxic monoclonal antibodies. This was further demonstrated through characterizing cytotoxic activities of antitumor monoclonal antibodies of different murine IgG subclasses (2). When the variable region of the anti-gp75 TA99 monoclonal antibody clone was produced with different IgG subclass scaffolds, the cytotoxic effect of the monoclonal antibody was found to directly correlate with the A/I ratio. The mouse IgG2a subclass that has the highest A/I ratio demonstrated the most potent *in vivo* activity. In preclinical studies, activating Fc $\gamma$ R-expressing monocytes and/or macrophages, but not NK cells, were shown to be the primary effector cell types mediating elimination of tumor cells by therapeutic monoclonal antibodies (65–68). A similar role for macrophages in humans is still a matter of debate and needs to be elucidated. The requirement of Fc-Fc $\gamma$ R interactions and the impact of the binding affinity of the IgG Fc to activating Fc $\gamma$ Rs have been confirmed in humans by comparing the response to these therapeutics in patients expressing high- or low-binding alleles of Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa. Expression of higher-affinity Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa alleles predicted enhanced response rate among lymphoma patients treated with rituximab (69, 70), breast cancer patients treated with trastuzumab (71), and metastatic colorectal cancer patients treated with cetuximab (72).

These animal and clinical studies provided motivation for the development of a second generation of cytotoxic antibodies that exploit Fc-Fc $\gamma$ R pathways to improve clinical efficacy. Indeed, Fc engineering to increase the A/I ratio of therapeutic IgG1 antibodies, either by point mutations at the Fc domain (13) or by modifying the composition of the Fc-associated glycan (73), is an effective strategy to yield therapeutic antibodies with increased potency. In preclinical animal studies, enhanced *in vivo* activity was observed when monoclonal antibodies targeting melanoma-associated differentiation antigen gp75, CD19, and CD20 were Fc-engineered through point mutations to enhance affinity for activating Fc $\gamma$ Rs (12, 13, 74). Similarly, a bisected, afucosylated, Fc-glycoengineered anti-EGFR monoclonal antibody demonstrated enhanced ADCC activity and resulted in superior *in vivo* efficacy compared to cetuximab, a nonglycoengineered anti-EGFR monoclonal antibody (75). This approach has been advanced to the clinic with the FDA approval of obinutuzumab, an afucosylated anti-CD20 monoclonal antibody that, when combined with chemotherapy, extended progression-free survival of patients with chronic lymphocytic leukemia compared to the wild-type IgG1 rituximab plus chemotherapy. Based on these preclinical and clinical studies, many next-generation, Fc-optimized antitumor monoclonal antibodies have been developed and are currently being evaluated for clinical use.

While the clearance of tumor cells is readily achieved by antitumor monoclonal antibodies, evidence has accumulated suggesting that a long-term cellular memory vaccinal response can also be induced as a consequence of cytotoxic monoclonal antibody therapy. Studies using mouse models have demonstrated that CD20 monoclonal antibody treatment induces antitumor T cell responses (12, 76). Similar effects in humans are supported by the long-lasting responses observed in some patients after single-course treatment with rituximab and by the presence of tumor antigen-specific T cell responses in some B cell lymphoma patients treated with rituximab (77, 78). A vaccinal effect has also been characterized in patients with HER2- and MUC1-expressing tumors who can develop specific T cell responses after treatment with anti-HER2 and anti-Mucin-1 (MUC1) monoclonal antibodies (79, 80). Recent studies have elucidated the involvement of Fc $\gamma$ R pathways in the induction of antitumor vaccinal effects. Using human CD20 as a tumor neoantigen, antihuman CD20 monoclonal antibody treatment in wild-type and in Fc $\gamma$ R knockout mouse strains revealed that both the primary clearance of the CD20-expressing tumor cells and the

generation of memory T cell responses were dependent on Fc $\gamma$ R interactions (12, 76). Studies in Fc $\gamma$ R-humanized mice showed that the initial depletion of tumors by cytotoxic effector cells was mediated by Fc $\gamma$ RIIIa on monocytes and macrophages, whereas the formation of a memory T cell response was dependent on Fc $\gamma$ RIIa expression on dendritic cells. Overall, this work (12) suggested a mechanism whereby administration of an anti-CD20 monoclonal antibody triggered macrophage-mediated killing of tumor cells through Fc $\gamma$ RIIIa, leading to IgG immune complex formation and engagement of activating Fc $\gamma$ RIIIa on dendritic cells; this engagement stimulated dendritic cell activation, maturation, and cross-presentation of tumor neoantigens to T cells, leading to the induction of antitumor T cell memory. These studies (12) suggest novel strategies for third-generation antitumor antibodies in which Fc engineering is designed to enhance not only cytotoxicity but also T cell memory responses by appropriate Fc $\gamma$ R engagement.

### **Immunomodulatory Antibodies**

Cancer therapy has been revolutionized with the development of immune checkpoint-blocking monoclonal antibodies targeting the inhibitory T cell signaling receptors CTLA-4 (cytotoxic T lymphocyte-associated protein 4) and PD-1 (programmed death-1) (81). Clinical studies have supported the use of these monoclonal antibodies either as monotherapies or in combination, thus validating the approach of targeting immune cells with specific monoclonal antibodies (82–84). These monoclonal antibodies act by targeting coregulatory receptors on the surface of immune cells in order to enhance antitumor immune responses. Mechanistically, immunomodulatory monoclonal antibodies act as agonists or antagonists to stimulate or block, respectively, a target antigen. It is now clear that many of these therapeutic monoclonal antibodies depend on Fc-Fc $\gamma$ R interactions to achieve full therapeutic activity. Animal studies have highlighted the importance of activating type I Fc $\gamma$ Rs for the antitumor activity of several immunomodulatory monoclonal antibodies, such as anti-CTLA-4 (85–87), GITR (glucocorticoid-induced TNFR-related protein) (87), OX40 (88), and PD-L1 (PD ligand 1) (89). These studies elucidated unexpected Fc $\gamma$ R-dependent depletion of specific regulatory immune cell populations in the tumor microenvironment that provided therapeutic modulation of the immune cell composition at tumor sites. Thus, independent of their Fab-mediated mechanism aiming to either block or activate inhibitory or stimulating T cell signals, these monoclonal antibodies act to deplete target immune cells in an activity similar to that of cytotoxic tumor-targeting monoclonal antibodies described above. More specifically, the antitumor activity of anti-CTLA-4, GITR, and OX40 monoclonal antibodies is significantly augmented by the depletion of regulatory T cells (Tregs) within the tumor microenvironment (90). Despite the wide distribution and expression of these receptors on different T cell compartments, monoclonal antibody-mediated targeting and elimination are preferentially directed to intratumoral Tregs. Treg depletion increases the ratio of effector T cells (Teffs) to Tregs in the tumor microenvironment, which favors efficient tumor clearance. Simpson et al. (85) demonstrated that this specific effect of anti-CTLA-4 monoclonal antibody depends on two main factors that distinguish the tumor microenvironment from lymphoid organs. First, Tregs have higher expression density of surface CTLA-4 compared to Teffs, and CTLA-4 expression level is elevated in the tumors compared to lymphoid organs. Thus, higher receptor expression density preferentially targets Tregs for ADCC. Second, depletion of Tregs by CTLA-4 monoclonal antibodies is dependent on Fc $\gamma$ RIV-expressing macrophages, which are more abundant and express higher levels of Fc $\gamma$ RIV at the tumor site compared to the draining lymph nodes. By characterizing the activity of different mouse IgG subclasses, Selby et al. (86) further demonstrated the requirement for an Fc scaffold with a high A/I ratio in order to mediate the therapeutic activity of CTLA-4 monoclonal antibodies. Similar requirements for receptor overexpression by intratumoral Tregs

and the presence of myeloid cells expressing activating FcγRs in the tumor microenvironment have been characterized as a general mechanism for additional immunomodulatory monoclonal antibodies. The therapeutic activity of monoclonal antibodies targeting the activating receptors GITR and OX40 depends on their ability to modulate the tumor microenvironment by depleting intratumoral Tregs. An alternative mechanism to immunomodulate the tumor microenvironment has been described for anti-PD-L1 monoclonal antibodies, which target the ligand for PD-1 (89). PD-L1 monoclonal antibodies were found to have superior antitumor activity when engaging activating FcγRs to deplete intratumoral myeloid cells and thereby modulating myeloid cell populations in the tumor microenvironment. This FcγR-dependent mechanism synergized with the FcγR-independent blocking of the inhibitory PD-1/PD-L1 axis for optimal therapeutic efficacy.

Evidence for Treg depletion was recently demonstrated in small groups of melanoma patients treated with ipilimumab, the FDA-approved anti-CTLA-4 IgG1 monoclonal antibody. In these patients, a higher baseline frequency of peripheral nonclassical monocytes and tumor-associated macrophages that expressed FcγRIIIa was associated with treatment responsiveness. Furthermore, these nonclassical monocytes were shown to mediate Treg depletion by ipilimumab *ex vivo* (91). A second human anti-CTLA-4 monoclonal antibody that has been used clinically is tremelimumab, which is an IgG2 antibody and therefore has significantly reduced FcγRIIIa binding affinity relative to IgG1 and would be expected to mediate reduced ADCC activity. Despite failing to show any benefit over chemotherapy in a phase III study, tremelimumab exhibits some therapeutic activity in combinational trials assessing tremelimumab in combination with other anticancer drugs (92). This activity may reflect the multiple mechanisms that can be induced by CTLA-4 monoclonal antibodies in humans. Further studies on these therapeutic monoclonal antibodies are needed to fully exploit their mechanism of action in patients. Interestingly, PD-L1 monoclonal antibodies that demonstrate clinical activity are either human IgG4, which have low FcγR binding and ADCC activity, or an Fc-null version mutated to abrogate FcγR binding. One PD-L1 monoclonal antibody that has recently completed phase I trials, avelumab, is a human IgG1 isotype and would therefore be predicted to mediate ADCC *in vivo*. It remains to be evaluated in advanced human trials whether therapeutic activity of avelumab is enhanced through engagement of FcγRs, as has been observed in preclinical models using other anti-PD-L1 antibodies.

Recent studies revealed an unexpected requirement for engagement of the inhibitory FcγRIIb for optimal agonistic activity of monoclonal antibodies targeting members of the TNFR superfamily, including CD40, DR5, CD30, and Fas (93–96). Contrary to antitumor cytotoxic and immunomodulatory monoclonal antibodies that require activating FcγR engagement and signaling to mediate specific elimination of target cell populations, engagement of the inhibitory FcγRIIb by anti-TNFR monoclonal antibodies does not require FcγR intracellular signaling to mediate IgG agonistic activity. FcγRIIb potentiates the activity of agonistic monoclonal antibodies by solely providing a scaffold, *in trans*, for antibody cross-linking, enhancing thereby target receptor clustering on the cell surface. FcγRIIb engagement is mainly required for the activity of monoclonal antibodies targeting receptors that depend on multimerization for their activation, such as members of the TNFR family. Whereas soluble, non-cross-linked monoclonal antibodies fail to mimic the trimeric TNFR-TNF complex needed for activation, FcγRIIb-cross-linked monoclonal antibodies have the capacity to confer potent agonistic activity *in vivo*. A similar requirement for FcγRIIb engagement was observed for the agonistic activity of monoclonal antibodies targeting a member of the class A scavenger receptor family (MARCO; macrophage receptor with collagenous structure) (97). Anti-MARCO monoclonal antibodies mediate reprogramming of MARCO-expressing tumor-associated macrophages from a protumoral, immunosuppressive M2 phenotype to an M1-like one, modulating the tumor microenvironment into a more inflammatory and antitumoral state. Similar to anti-TNFR monoclonal antibodies, FcγRIIb engagement

mediates oligomerization of the MARCO collagenous domains, inducing thereby intracellular signaling and downstream effects on macrophage polarization.

Given the requirement of these antibodies for Fc $\gamma$ RIIb engagement, Fc $\gamma$ RIIb-targeted Fc engineering could enhance their *in vivo* antitumor activity. CD40 is perhaps the most studied therapeutic target for the Fc $\gamma$ R-dependent cross-linking pathway. Agonistic anti-CD40 monoclonal antibodies represent physiologic adjuvants that function by potently ligating CD40 to induce APC maturation and subsequent expansion of tumor-specific cytotoxic T cells that have the capacity to efficiently eradicate tumors in multiple models (98). Indeed, studies in Fc $\gamma$ R-humanized mice revealed that agonistic anti-CD40 antibodies optimized for enhanced human Fc $\gamma$ RIIb binding displayed a remarkable (more than tenfold) improvement in their immunostimulatory and antitumor activity (13, 93). Likewise, further studies on agonistic antibodies targeting other TNFR superfamily members, such as DR5, also showed that their activity can be enhanced through Fc $\gamma$ RIIb-targeted Fc engineering (94, 95). Several antihuman CD40 antibodies have been developed, and among them, CP-870,893 is the most potent agonistic antihuman CD40 monoclonal antibody reported to date (99). Despite its potent antitumor activity in preclinical animal models, CP-870,893 displayed only modest antitumor activity in patients with tumors in a series of phase I clinical trials (99–101). Using mice fully humanized for both CD40 and Fc $\gamma$ Rs, the modest activity of CP-870,893 was shown to be a function of the limited capacity of human IgG2 to interact with human Fc $\gamma$ RIIb (102). Indeed, subclass switching of CP-870,893 to human IgG1 significantly improved its *in vivo* activity, owing to the increased affinity of human IgG1 for Fc $\gamma$ RIIb. Likewise, Fc domain engineering to selectively enhance affinity of the IgG1 Fc for Fc $\gamma$ RIIb further improved the *in vivo* antitumor activity of CP-870,893, indicating that Fc variants specifically enhanced for Fc $\gamma$ RIIb binding may be the optimal IgG framework for agonistic anti-CD40 monoclonal antibodies. This work further highlights the utility of animal models humanized for both the target antigen and Fc $\gamma$ Rs to evaluate the contributions of Fab- and Fc-mediated mechanisms to the *in vivo* activity of human therapeutic antibodies and to guide the development of Fc-optimized clinical candidates.

## IgG SIGNALING IN INFECTION

The critical role of antibodies in host protection against infectious diseases is manifest by recurrent infections in patients with X-linked agammaglobulinemia (XLA), a primary immunodeficiency caused by a tyrosine kinase mutation that disrupts B cell differentiation, maturation, and signaling. XLA patients are typically healthy in infancy until maternal antibodies wane, after which time they become agammaglobulinemic and suffer tremendous infectious morbidity in the absence of replacement immunoglobulin therapy. Lifelong IVIG treatment complements the immune deficiency and enables most XLA patients to lead healthy, full lives.

Because IgG-mediated protection is often linked to recruitment of Fc $\gamma$ R-mediated effector functions, genetic variation in Fc $\gamma$ Rs can underlie differences in susceptibility to infectious diseases. For example, the low-affinity variant of Fc $\gamma$ RIIa (R131) confers increased susceptibility to severe bacterial infections and sepsis, likely because of impaired microbial clearance (37, 103, 104). In contrast, in dengue infection, where antibody-mediated enhancement (ADE) of virus infectivity can occur, this low-binding Fc $\gamma$ RIIa polymorphism is associated with asymptomatic infection and improved clinical outcomes (105, 106). IgG-mediated immunity against infection occurs through numerous mechanisms involving Fc $\gamma$ R-dependent and -independent mechanisms. *In vitro* assays have been useful in defining the various IgG-mediated mechanisms of protection, such as virus neutralization (107, 108), opsonophagocytic killing of bacteria (109), and killing of infected cells (110), but mechanisms that play a role in *in vivo* protection cannot be reliably predicted from

in vitro experiments. Here, we review the mechanisms of in vivo IgG-mediated protection using studies of immunity to influenza viruses and to HIV as model systems.

## Influenza

Neutralizing antibodies against influenza viruses bind the HA, which is responsible for mediating viral entry into host cells. Anti-HA neutralizing antibodies work by blocking one of the two key functions of the HA that result in viral entry—attachment to host cells or fusion with the endosome, which is required for release of viral RNA into the cytoplasm (111, 112). The two types of anti-HA neutralizing antibodies (those that prevent attachment or fusion) can be distinguished in vitro using the classic hemagglutination inhibition (HI) assay, which detects neutralizing antibodies that act by preventing attachment of HA to sialic acids, or using cell-based microneutralization assays, which detect neutralizing antibodies that act by either mechanism. Neutralizing antibodies that prevent attachment bind to specific antigenic sites surrounding the receptor binding pocket in HA and are characterized by potency and prompt selection of viral escape variants. Escape in the presence of these neutralizing antibodies occurs for three reasons: (a) The influenza virus polymerase is a low-fidelity complex lacking exonuclease activity that generates a high rate of transcriptional mutation relative to other RNA polymerases, (b) the rapid kinetics of influenza virus replication results in efficient selection of escape variants when selective pressure of a neutralizing antibody is applied, and (c) the ability of antigenic sites in the HA to accommodate tremendous structural diversity means that the virus can nearly always escape neutralizing antibodies that bind to those regions. Because the neutralizing antibodies that prevent viral attachment readily select for escape variants, they are, by nature, strain specific (113). In vivo, neutralizing antibodies that prevent attachment are generally protective at very low concentrations, and those that have been studied protect without a requirement for FcγR engagement (35).

Neutralizing antibodies that prevent fusion are characterized by binding to highly conserved domains of the HA, such as the stalk domain, and they are usually of lower protective potency in vivo. Because fusion-inhibiting neutralizing antibodies bind regions of the HA that cannot accommodate as much antigenic change as antigenic sites, they can demonstrate breadth of protective activity against different influenza viruses that is not seen with neutralizing antibodies that prevent viral attachment (107). New influenza vaccine candidates designed to elicit broadly neutralizing antibodies often aim to elicit these fusion-inhibiting neutralizing antibodies because of the breadth of protection that they can mediate in vivo. Several fusion-inhibiting neutralizing antibodies have been studied for FcγR requirements and were shown to protect without a requirement for FcγR engagement at relatively high concentrations, but activating type I FcγRs were required for their activity at lower concentrations (114, 115). Thus, potency of broadly-neutralizing antibodies is dependent on type I FcγRs in vivo.

In contrast to neutralizing antibodies, which can confer sterilizing immunity against influenza viruses, nonneutralizing antibodies specific for the influenza HA, neuraminidase, nucleoprotein, or M2 proteins can also contribute to protection by limiting virus replication and spread. Antibodies against the viral neuraminidase, for example, can function in several ways, including inhibiting neuraminidase activity that is required for release of new virus particles from the host cell, or they can mediate cytotoxic activity of infected cells (116). Like the fusion-inhibiting neutralizing antibodies, nonneutralizing antibodies can be broadly reactive and broadly protective in vivo; they are generally of lower protective potency than the neutralizing antibodies that prevent viral attachment, and several broadly reactive anti-HA or neuraminidase monoclonal antibodies have been shown to be dependent on type I FcγRs for potent in vivo protection (114). The cumulative data support a model where strain-specific neutralizing antibodies do not require recruitment of

Fc $\gamma$ R-mediated effector functions, whereas broadly protective antibodies depend on activating type I Fc $\gamma$ R interactions for potent activity *in vivo*.

Interestingly, in addition to the varying requirements of different anti-influenza IgGs for Fc $\gamma$ Rs to mediate protection *in vivo*, different categories of protective IgGs also appear to have distinct capacities for recruiting cellular effector functions. For example, a few neutralizing antibodies that have been studied and that prevent viral attachment do not readily engage Fc $\gamma$ RIIIa without Fc engineering to increase Fc-Fc $\gamma$ RIIIa binding affinity and therefore cannot mediate ADCC in *in vitro* assays (115). This appears to be inconsequential *in vivo*, as they protect—potently—without a requirement for Fc $\gamma$ Rs. In contrast, however, neutralizing antibodies and monoclonal antibodies with broad protective activity *in vivo* do generally require Fc $\gamma$ R engagement, and their Fcs are able to engage Fc $\gamma$ RIIIa to mediate ADCC *in vitro* (114, 115). Determinants of Fc $\gamma$ R engagement by bound IgGs are not fully understood, but they include binding density, which is dictated by the nature and expression of antigen, and may also be affected by affinity of the Fab for binding epitope; IgGs with moderate-affinity Fab domains have been shown to recruit effector function more effectively when compared to IgGs with higher-affinity Fab domains. This has been hypothesized to result from the increased off-rates with lower-affinity Fab-antigen interactions, leading to more monovalent Fab-antigen interactions and increased potential for dense IgG opsonization (one Fc domain per bound Fab domain). In contrast, higher-affinity IgGs are more likely to form bivalent interactions with antigen (one Fc domain per two bound Fab domains), limiting the potential for dense IgG binding (117). ADCC mediated by broadly reactive anti-HA monoclonal antibodies may also be supported through increased affinity between target and effector cell owing to broadly neutralizing monoclonal antibody Fc-Fc $\gamma$ RIIIa engagement in conjunction with binding by the viral HA to sialic acids on the effector cell (118). Yet another mechanism that may affect the ability of a bound IgG to engage Fc $\gamma$ Rs is conformational change in the Fc domain induced upon binding of the Fab domain to antigen (119–121). In this model, some epitope-paratope interactions induce structural change in the Fc domain that affects Fc $\gamma$ R interactions. A correlate to this is that IgG constant domains can affect fine specificity of the variable domain; this has been demonstrated in studies showing that identical variable domains in IgGs of distinct subclasses can have different binding specificities (122).

An area for further investigation in Fc $\gamma$ R signaling and influenza biology is the extent to which effector functions mediated by activating type I Fc $\gamma$ Rs can exert selective pressure on influenza viruses *in vivo*. The seasonal antigenic drift that dictates constant updating of virus strains in current influenza vaccines occurs under Fc $\gamma$ R-independent selective pressure by neutralizing antibodies that block virus attachment. Because approved influenza virus vaccines do not aim to elicit broadly reactive antibodies, it is not clear how a new generation of vaccines that may provide broad immunity will affect the antigenic landscape of circulating influenza viruses. Vaccines that elicit broadly reactive HA or neuraminidase IgGs will likely protect *in vivo* largely through Fc $\gamma$ R-dependent mechanisms, meaning cellular effector functions will become the driving force behind seasonal antigenic drift. The rate of antigenic drift and effects on viral fitness that will be observed in viruses that are selected under pressure of broadly protective IgGs will be important to understand as new influenza vaccines are studied and brought to market.

## HIV

Neutralizing antibodies against HIV are specific for the major envelope glycoprotein (Env) that mediates viral entry. As with the influenza HA, epitopes of the HIV envelope protein that can mediate potent virus neutralization are often able to accommodate tremendous structural diversity, making broadly neutralizing anti-HIV IgGs extremely rare. A small fraction of HIV-infected

individuals, however, do develop affinity-matured IgGs with broad protective activity against distinct virus isolates (123), demonstrating potential for broad in vivo protection against HIV-1 through passively transferred anti-Env IgGs. Advances in B cell cloning technology have enabled screening of single B cells from these HIV patients; rare cells producing broadly neutralizing IgG specificities have been isolated, and the human antibodies have been cloned and characterized (123). In vivo studies have demonstrated that sterilizing immunity against SHIV infection can be achieved through passive administration of broadly neutralizing anti-Env monoclonal antibodies in macaques and against HIV-1 infection in humanized mice (124–126). Recent landmark clinical studies have shown that administration of a broadly neutralizing anti-Env monoclonal antibody that disrupts interaction with CD4 can suppress viremia in patients with chronic HIV-1 infection (127).

Mechanistic studies have demonstrated increased in vivo activity of anti-Env neutralizing antibodies with capacity for enhanced binding to activating type I FcγRs in preventing HIV-1 entry, suggesting a role for activating FcγRs in in vivo neutralization (33). Similarly, activating FcγRs have been shown to mediate in vivo clearance of HIV-infected cells, further contributing to the antiviral activity of anti-Env neutralizing antibodies (128). Further assessment of the protective activity of Fc variants of anti-Env monoclonal antibodies with differential FcγR binding capacities in postexposure prophylaxis and therapy models of HIV-1 infection in humanized mice revealed a requirement for activating FcγR engagement for optimal therapeutic activity of anti-Env neutralizing antibodies (33, 129). These findings are consistent with previous observations of a nonhuman primate pre-exposure prophylaxis model in which Fc domain variants with minimal capacity for FcγR engagement provided minimal protection against SHIV challenge (125). Importantly, these studies demonstrated that IgGs can mediate suppression of a chronic viral pathogen, HIV-1, and that activating type I FcγRs are required for this activity in vivo. Given the contribution of FcγR-mediated pathways in the in vivo antiviral activity of anti-Env neutralizing antibodies, Fc domain engineering for selective engagement of activating FcγRs is expected to lead to the development of anti-Env neutralizing antibodies with improved therapeutic efficacy.

## Other Infectious Diseases

Studies support an important role for FcγR signaling in protection against a number of pathogens in addition to influenza viruses and HIV. For example, passive transfer of an anti-RSV (respiratory syncytial virus) monoclonal antibody, palivizumab, with enhanced affinity for the activating FcγRIIIa by virtue of modification with afucosylated Fc glycoforms, reduced RSV lung titers in vivo compared with the fucosylated version; abrogation of palivizumab-FcγR binding further reduced in vivo protection, demonstrating that FcγR-independent neutralization plays little role in protection mediated by palivizumab in vivo (130). Protection against some bacterial and fungal pathogens has also been shown to depend on FcγRs. FcγRIIIa was shown to be required for monoclonal antibody-mediated protection against *Streptococcus pneumoniae*, and numerous experiments have shown that subclass determines protective activity of IgGs against *Staphylococcus aureus* and *Cryptococcus neoformans*, with affinity for activating FcγRs correlating with improved protection (reviewed in 131). Based on these studies, monoclonal antibodies engineered for selectively enhanced binding to activating FcγRs have been developed as passive transfer therapeutics; the in vivo activity of a monoclonal antibody against the lethal toxin of *Bacillus anthracis*, for example, was improved through Fc optimization that enhanced binding to activating human FcγRs (34), and, as described above, similarly engineered monoclonal antibodies have shown increased potency in suppression of HIV rebound (33, 129). In addition to activating FcγRs, signaling through the inhibitory FcγRIIb can also affect susceptibility to infections, since it offsets activating FcγR effects. For example, *Fcgr2b*<sup>-/-</sup> mice exhibit improved bacterial clearance in models of

pneumococcal peritonitis through improved macrophage effector function (132, 133), whereas overexpression of Fc $\gamma$ RIIb is associated with increased mortality upon challenge with *S. pneumoniae* (132, 133). Collectively, these findings demonstrate the importance of IgG–Fc $\gamma$ R-mediated effector functions in protection against a diverse set of infectious organisms in vivo.

### **Antibody-Dependent Enhancement of Infection and/or Disease**

A long-appreciated, yet poorly understood, property of type I Fc $\gamma$ Rs is their occasional ability to mediate ADE of infectivity and disease. An example of ADE can be observed in secondary infection with dengue virus; although primary infection is often asymptomatic or mild in presentation, a second infection with a heterologous dengue serotype can be accompanied by increased viral replication and severe disease. This enhancement is also seen, on occasion, in infants with primary dengue infection who have maternal antidengue antibodies that bind, but do not neutralize, the infecting virus strain (134, 135). ADE in secondary dengue is thought to be mediated by cross-reactive, non-neutralizing IgGs generated during the primary virus exposure that increase infectivity of type I Fc $\gamma$ R-bearing cells, including monocytes, macrophages, and dendritic cells. ADE of dengue virus infectivity and/or disease has been demonstrated in a variety of in vitro and in vivo infection models; studies have shown that dengue virus infection in the presence of dengue-binding, but nonneutralizing, IgGs can result in more infected cells (136–138), enhanced virus fusion activity (139), and/or suppression of innate immunity (140, 141), but specific Fc $\gamma$ R pathways required for ADE in dengue infection have not been identified (142–145). Interestingly, selectivity of patient IgG responses may play a role in susceptibility to severe dengue disease and explain why only a small fraction of patients with preexisting, nonneutralizing antidengue IgGs develop severe disease (146). Analysis of IgGs from patients with mild or severe dengue disease showed that patients with severe disease had Fc structures on antidengue IgGs that were selectively enhanced for Fc $\gamma$ RIIIa binding (T.T. Wang and J.V. Ravetch, unpublished observation). This suggests that production of IgGs with higher affinity for Fc $\gamma$ RIIIa may play a role in ADE of disease in dengue infection.

ADE of disease severity not due to increased microbial replication may also occur in some settings owing to formation of pathological immune complexes that trigger enhanced inflammation. This mechanism is not well understood, but some speculate that pathological immune complexes were responsible for failure of an RSV vaccine trial in 1968 in which children who received the experimental vaccine were more than 30 times more likely to be hospitalized with severe RSV infection compared with control subjects and two toddlers died as a result of what was thought to be vaccine-enhanced disease (147). The experimental vaccine was a formalin-killed RSV that elicited a large proportion of nonneutralizing antibodies that are thought to have formed insoluble immune complexes with viral proteins that may have mediated cytotoxicity and/or triggered cytokine production, leading to enhanced disease (148–150). Disease exacerbation through immune complex deposition was also proposed for patients with fatal infections of 1957 or 2009 pandemic influenza who were found to have large quantities of immune complexes deposited in pulmonary tissue (148).

How some microbes cause increased infectivity and/or clinical disease through antibody-mediated mechanisms is not well understood, but the phenomenon likely relies on both host and pathogen determinants. Adaptation to productive replication in activating Fc $\gamma$ R-bearing cells such as macrophages and monocytes may be one determinant of ADE. A second determinant could be the antigenic variability of a pathogen, because repeated exposures can lead to the presence of cross-reactive, nonneutralizing IgGs, which may mediate pathogen uptake by Fc $\gamma$ R-expressing cells or, in rare circumstances, may form immune complexes that enhance inflammation and disease due to complement-mediated inflammation, type I Fc $\gamma$ R-mediated inflammation, or direct cytotoxicity.

## CONCLUSIONS

IgG antibodies are a major component of the adaptive immune system and have the capacity to mediate pleiotropic effector activities through specific interactions with type I and type II FcγRs. In addition to IgG antibodies playing a direct role in protection against malignancies and infections, IgG-mediated signaling has a key role in shaping adaptive immune responses. This is because antigen is often recognized *in vivo* as IgG immune complexes because IgG antibodies that were preexisting or that are produced early during an adaptive response bind to the antigen during infection or vaccination.

For example, one mechanism by which these immune complexes modulate adaptive immunity is by enhancing T cell responses, as described above. Cross-linking of activating type I FcγRs on dendritic cells triggers their maturation and leads to enhanced antigen presentation and activation of T cell responses. This phenomenon is exemplified by *in vivo* work showing that formation of a protective memory T cell response (vaccinal response) after rituximab administration is dependent on rituximab/CD20 immune complex formation and expression of FcγRIIa on dendritic cells (12). IgG immune complexes can also act in an autocrine signaling manner to modulate selection of B cells during maturation of an antibody response. This was recently demonstrated *in vivo* in studies showing that sialylated immune complexes signal through the type II FcγR, CD23, on B cells to trigger upregulation of the B cell inhibitory FcγRIIb. Increased FcγRIIb expression elevates the threshold for selection of B cells based on affinity of the BCR, ultimately resulting in selection for affinity-matured cells (7). Interestingly, it was also shown that the initial step in this autocrine signaling cascade, engagement of CD23, is regulated during maturation of antibody responses in humans; Fc sialylation on antigen-specific IgGs was shown to increase in the days following influenza virus vaccination, with the increased change in sialylation correlating with final affinity of vaccine response. Immunization with sialylated immune complexes was shown, in an influenza system, to result in higher-affinity IgG responses with enhanced *in vivo* potency and breadth of protective activity (7). These findings highlight the interplay of different types of FcγRs in mediating effector activities and modulating adaptive immune responses. Fc domain engineering of therapeutic antibodies to engage particular classes of FcγRs substantially improved their clinical efficacy. Given the contribution of Fc-FcγR interactions to the modulation of adaptive immune responses, specific activation of FcγR-mediated pathways during vaccination could also lead to the generation of robust and sustained adaptive immune responses, with broad and potent protective activity.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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