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Signaling by Antibodies: Recent Progress

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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 pro-inflammatory, 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 vaccine responses.

Keywords

IgG; Fc receptors; effector function; Immunomodulation; Immunity; Inflammation

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 γ receptors; Fc γ 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 γ 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 γ 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 γ R interactions and, in turn, Fc γ R aggregation and signaling. The specific Fc γ 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

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domain of the Fc (Figure 1A) (1, 2). On the basis of binding either an open or closed conformation taken by the Fc domain, Fc γ Rs can be distinguished as Type I or II, respectively. These two receptor families transduce distinct effector and modulatory pathways and 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 inter-chain disulfide bridges. The subclass of IgG is determined by protein sequence of the heavy chain, with all subclasses pairing with either κ or λ light chains. A basic Y-shaped structure is common to all IgG subclasses and results from a hinge that separates the CH1 and light chains, comprising the Fab domain, from the CH2 and CH3 domains that constitute the Fc domain (Figure 1A). While elaboration of a κ vs. λ 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 γ 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 over 90%, but differences that exist at the hinge-proximal CH2 domain, the interface of Fc-Fc γ R interactions, confer distinct biological activities to IgG subclasses by determining Fc γ R binding affinity. Each subclass is distinct in its ratio of binding to activating over inhibitory Fc γ Rs (A/I ratio), with human IgG1 and IgG3 or mouse IgG2a/2c having the highest A/I ratios. In addition to distinct Fc γ R binding affinities, the abundance of circulating IgG subclasses varies with IgG1 in highest abundance in humans, followed by IgG2>IgG3>IgG4.

Along with the heavy chain subclass, 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 γ R binding. The core structure of this glycan can be modified by additional saccharide units that determine specific Fc-Fc γ 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 γ Rs (3). Glycan structures lacking a core fucose residue enhance affinity for the activating Type I Fc γ RIIIa through stabilized glycan-glycan interactions between the Fc domain and Fc γ 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 individuals (TTW and JVR, 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's granulomatosis (9).

Type I and Type II Fc γ receptors

Type I Fc γ Rs belong to the immunoglobulin superfamily and share many structural and functional properties (Figure 1B). The extracellular domain of Type I Fc γ Rs is comprised of either two or three Ig domains and interacts with the hinge proximal region of the CH2 domain of IgG in a 1:1 stoichiometric complex. Type I Fc γ Rs share highly conserved

intracellular motifs that mediate signaling upon receptor crosslinking by IgG immune complexes, yet distinct functional outcomes of signaling define each receptor. Type I Fc γ R_s 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 Fc γ R_s are expressed in mice (Fc γ RI, Fc γ RIII, and Fc γ RIV) and humans (Fc γ RI, Fc γ RIIa, and Fc γ RIIIa), and a single inhibitory Fc γ R, Fc γ RIIb, is expressed in both species. Each Fc γ R has a distinct pattern of cellular expression (Table 1) with engagement of activating Fc γ R_s inducing positive mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and pro-inflammatory cytokine production. Signaling through the inhibitory receptor, Fc γ RIIb, results in negative functions such as inhibition of inflammatory immune responses. Fc γ RI is the only high affinity Fc γ R, capable of binding monomeric IgG (K_D 10^{-9} – 10^{-10} M for human IgG1); all other Type I Fc γ R_s 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 Fc γ R_s is determined by the IgG subclass and the Fc glycan composition, as described above (1).

Type II Fc γ R_s 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 Fc γ R_s with known functional activities include DC-SIGN (mouse orthologue SIGN-R1) and CD23, which share an oligomeric structure that is stabilized through an α -helical coiled-coil stalk motif at the extracellular receptor domain. The primary determinant of IgG binding to Type II Fc γ R_s is the presence of sialylated Fc glycoforms, which destabilize the Fc structure enabling assumption of a conformation that is the ligand for Type II Fc γ R_s. As with the majority of Type I Fc γ R_s, Type II Fc γ R_s 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), while 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 Fc γ R signaling pathways and downstream functions are described throughout this chapter.

Species specificity in IgG signaling

All known animal models, including non-human primates, express species-specific IgG subclasses and Fc γ R_s that are unique in structure, function and cellular distribution. Such inter-species variation limits interpretation of data from mixed-species systems, i.e. in experiments using human IgGs in non-human primates or in mice. While affinities between human IgGs and Fc γ R_s 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 Fc γ R_s 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 and mouse monocyte-derived dendritic cells. For example, human monocyte-derived dendritic cells express one activating Type I receptor, Fc γ RIIa, along with the inhibitory Fc γ RIIb, whereas mouse monocyte-derived dendritic cells express all murine Type I Fc γ Rs. Another example from non-human primates is found in neutrophil Fc γ R expression: while human neutrophils express Fc γ RIIa, Fc γ RIIb and Fc γ RIIIb, neutrophils from non-human primate species like rhesus macaques do not express Fc γ RIIIb but do express Fc γ RI in addition to Fc γ RIIa and Fc γ RIIb. 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 Fc γ RIV on macrophages; mouse NK cells do not express Fc γ RIV and thus do not significantly contribute to antibody mediated cellular depletion *in vivo* by IgG_a (12). In contrast, ADCC in humans is mediated through the human IgG1 subclass engaging Fc γ RIIIa, 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 Fc γ RIIIa expressed and the fucosylation state of the IgG1 Fc. The best model currently available for the *in vivo* study of human Fc γ Rs is a mouse model in which endogenous mouse Fc γ R genes were deleted and human Fc γ Rs are instead expressed under the control of their endogenous human promoters. This Fc γ R humanized mouse strain recapitulates the human Type I Fc γ Rs in form, function and cellular expression (13).

Interplay Between Type I and Type II Fc γ Rs in Regulating Immune Responses

Interactions between Fc γ Rs and the Fc domain of IgG antibodies activate signaling pathways that vary depending on the Fc γ R and cell type that is engaged; each Fc-Fc γ R 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 Fc γ Rs 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-Fc γ R interactions and subsequent signaling events, several factors influence the capacity of Fc γ R-mediated signaling to modulate immune processes. For example, each Fc γ R exhibits a distinct expression pattern among leukocyte populations, with different cell subsets commonly co-expressing more than one Fc γ R type (Table 1) (1, 13). Expression levels and, to a degree, the pattern of Fc γ R expression can fluctuate during leukocyte differentiation and activation, with each cell type generally co-expressing more than one Fc γ R type at any time (Table 1). Additionally, several Fc γ R genes can be induced acutely by chemokines and/or cytokines present at sites of inflammation, infection, or tissue injury, thereby influencing Fc γ R-mediated expression and, in turn, effector activities (11, 14, 15). The specific Fc γ R expression pattern on a cell determines whether receptor crosslinking will lead to cellular activation, ADCC and phagocytosis, release of cytokines and chemokines, leukocyte differentiation and survival, and/or modulation of T- and B-cell responses (16). In this section, we will focus on the key role of IgG antibodies in modulating immune signaling through selective engagement of different Fc γ Rs (Table 2).

Cytotoxicity of IgG-coated targets

All cytotoxic effector functions induced upon Fc γ R-IgG interactions are mediated through activating Type I Fc γ Rs. One of the first activities described for Fc γ Rs is their capacity to mediate clearance or cytotoxicity of IgG-coated targets, ranging from small antigens (toxins, infectious pathogens, etc.), to whole cells. Crosslinking of activating Type I Fc γ Rs 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 PI3K-PKC pathway and to Ca²⁺ 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 pro-inflammatory cytokines and chemokines with divergent effects on cellular survival and differentiation (19).

Although intracellular signals transduced upon receptor crosslinking are common to all leukocytes expressing activating Type I Fc γ Rs, the precise biological responses following Fc γ R engagement vary greatly between different leukocyte types. For example, granulocytes mediate biological effects within minutes following activation of Fc γ R-mediated pathways by IgG immune complexes (20). Granulocyte effector functions aim to destroy invading pathogens, often by releasing microbicidal molecules that are either preformed and stored in specialized cytoplasmic granules, or that are generated, de novo, upon Fc γ R engagement. For example, Fc γ R crosslinking leading to the activation of Syk and Src family kinases, triggers the production of reactive oxygen intermediates (ROI) through formation and activation of the NADPH-dependent oxidase complex (21, 22). Additionally, Fc γ R crosslinking induces activation of the PKC pathway and subsequent increase in intracellular Ca²⁺ stimulates mobilization and release of pre-formed cytotoxic or pro-inflammatory 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) as well as lipid mediators (leukotrienes) with potent pro-inflammatory activity (23-25). In addition, binding of multimeric IgG immune complexes to Fc γ Rs triggers receptor crosslinking 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 ROI and cytotoxic proteolytic compounds represent the two main strategies that granulocyte employ to achieve cytotoxicity of IgG-coated targets.

Similar signaling processes are also initiated upon engagement of the activating Fc γ RIIIa on NK cells, which triggers cellular activation and degranulation. In an analogy to granulocyte activation and degranulation following Fc γ R crosslinking, engagement of Fc γ RIIIa on NK cells is associated with ITAM phosphorylation and rapid increase in the intracellular Ca²⁺ levels that triggers the release of granular content, which include perforin and granzymes

(29, 30). The release of these potent enzymes in close proximity to IgG-coated targets induces the formation of pores at the target cell membrane and stimulation of pro-apoptotic pathways that ultimately lead to cell death.

In contrast to granulocytes and NK cells, elimination of IgG-opsonized targets by macrophages is achieved primarily through phagocytic processes, and not through the immediate release of cytotoxic and cytolytic compounds. Indeed, several studies have shown that Fc γ 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 γ R-dependent process (31, 32). Similarly, in mice humanized for Fc γ 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 γ RIIIa, activating Type I Fc γ R signaling is always balanced on effector leukocytes by signaling through co-expressed inhibitory Fc γ RIIb; thus, Fc γ R-mediated effector functions are the result of cumulative signaling from activating and inhibitory Type I Fc γ Rs (2). For example, the *in vivo* activity of cytotoxic therapeutic antibodies depends on a signaling balance that is weighted towards activating Fc γ Rs (2, 12, 33-36). This was demonstrated in early experiments utilizing an antibody (TA99) against a melanosome protein, gp75, in which it was evident that tumor clearance required activating FcR engagement and IgG subclass or glycovariants of the antibodies with enhanced capacity to engage activating Fc γ 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 γ Rs in regulating IgG-mediated inflammation comes from genetic association studies of Fc γ R genetic variants. For several autoimmune and chronic inflammatory pathologies, disease susceptibility and/or severity is associated with genetic variants of Fc γ 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, SLE patients are enriched for the low binding alleles for Fc γ RIIa, Fc γ RIIIa, and Fc γ RIIb, as well as lower copy number of the *FCGR3B* gene, which is characterized by reduced Fc γ RIIb expression levels (37-40). These variants exhibit reduced clearance of IgG immune complexes, likely increasing tissue immune complex deposition and inflammation. In addition, low copy number of *FCGR3B* is associated with the induction of ectopic expression of the inhibitory Fc γ RIIb in NK cells, likely reducing cytotoxic responsiveness (40). In contrast, high affinity variants of Fc γ RIIIa (V158) and Fc γ RIIb (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 Fc γ R-IgG interactions in mediating cytotoxic effector responses, and highlight that balanced activating and inhibitory Fc γ R 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 Fc γ R_s follow an identical pattern of receptor crosslinking and oligomerization that ultimately leads to sequestration of IgG-Fc γ R complexes in intracellular vesicles for lysosomal degradation and antigen processing (26). Indeed, all Type I Fc γ R_s (with the exception of the Fc γ RIIb1 splice variant) have the capacity to mediate phagocytosis of IgG-opsonized targets (41). The efficiency of Fc γ R-mediated phagocytosis varies greatly between leukocyte types and is dependent on differentiation stage, cytoskeletal motility, cellular size, and expression levels of Type I Fc γ R_s and other receptors such as pattern recognition receptors. Although both activating and inhibitory Type I Fc γ R_s can mediate phagocytosis in myeloid phagocytes, internalization of IgG-opsonized targets through activating Fc γ R_s mediates more potent effector responses (42-44). For example, phosphorylation of ITAM domains after Fc γ R crosslinking leads to pro-inflammatory signaling that mediate cellular activation and pro-inflammatory cytokine and chemokine expression, which modulates leukocyte differentiation, functional activity, and survival. Uptake of IgG-coated targets through activating Fc γ R_s 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 Fc γ R-mediated internalization of immune complexes results in enhanced T-cell activation (36, 46-48).

Apart from the direct effects on antigen uptake, processing and presentation to MHC class-II molecules, pro-inflammatory signals following Type I Fc γ R engagement by IgG immune complexes also influence several aspects of the activity and function of antigen-presenting cells. For example, signaling through Fc γ R_s 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 Fc γ R_s: the inhibitory Fc γ RIIb and the activating Fc γ RIIa (1). Since these receptors are co-expressed on the surface of dendritic cells, pro-inflammatory signals from Fc γ RIIa are antagonized by the inhibitory signaling activity of Fc γ RIIb. This balance between activating and inhibitory Fc γ R_s 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 Fc γ R_s are tightly regulated under homeostatic conditions, whereas certain mediators present at the inflammatory milieu can influence the expression of Fc γ RIIb or induce expression of additional activating Type I Fc γ R_s, altering thereby the threshold for IgG-mediated dendritic cell maturation. For example, IFN- γ induces the expression of the activating Type I Fc γ R, Fc γ RI and downregulates Fc γ RIIb expression. In contrast, IL-4 stimulates the expression of the inhibitory Fc γ RIIb (11, 14, 15, 48, 50). This finely-tuned interplay between activating and inhibitory Fc γ R expression determines the threshold for Fc γ R-mediated dendritic cell maturation and responsiveness to other stimuli (e.g. TLR ligands). In the absence of co-stimulatory signals, stimulation of dendritic cells with IgG immune complexes often fails to induce robust cell maturation. Activation of pro-inflammatory signaling pathways through innate immune receptors is generally required to overcome the inhibitory activity of Fc γ RIIb and induce dendritic cell activation (12, 36, 46, 48, 50).

provide anti-inflammatory activity in models of IgG-mediated inflammation (10, 11). These studies clearly highlight the importance of Type I and Type II Fc γ R signaling in the regulation of the activity and differentiation of antigen-presenting cells, 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 γ Rs is coupled to expression of the inhibitory Fc γ RIIb. In contrast, B cells constitutively express Fc γ RIIb throughout their development, without co-expressing any activating Type I Fc γ Rs. Fc γ 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 γ RIIb induces pro-apoptotic signals, whereas Fc γ RIIb – BCR co-engagement 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 pro-survival signals (56). Transition of B cells to plasma cells is associated with loss of BCR expression. In the absence of opposing BCR signaling, Fc γ 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, pro-apoptotic signaling activated through engagement of B-cell Fc γ RIIb alone is attenuated by co-aggregation with BCR. At the same time, thresholds for B cell activation through BCR are determined by *cis*-signaling through Fc γ RIIb, which can occur when antigen within an IgG immune complex binds BCR and Fc domains within the same complex co-engage Fc γ RIIb. Immune complex engagement of Fc γ RIIb leads to phosphorylation of the ITIM domains of Fc γ RIIb, in turn recruiting 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 γ and BTK and blocking induction of downstream signaling pathways (55, 56, 58). Studies using Fc γ RIIb-deficient mice have revealed how critical the contribution of Fc γ 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 γ 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 γ 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 γ RIIb with SLE (62). This polymorphism affects receptor partitioning to lipid raft membrane microdomains, thus diminishing Fc γ RIIb signaling activity. This central role of Fc γ RIIb in regulating BCR signaling activity represents a key determinant in the generation of antibody responses, B-cell selection and affinity maturation, as well as maintenance of peripheral tolerance (49, 55, 56, 59).

Apart from the Type I Fc γ RIIb, B cells express the Type II Fc γ 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⁺ APCs, leading to enhanced CD4⁺ T-cell immunity (63). As CD23 has also 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 γ RIIb, thus elevating the threshold for B-cell selection, resulting thereby 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^{-/-} 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 Fc γ R 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 will review the role and function of Type I and Type II Fc γ R in the *in vivo* protective activities of these antibodies. Numerous monoclonal antibodies (mAbs) with antitumor or antimicrobial activity are currently in development or clinical use, representing promising therapeutic modalities for the treatment of several infectious diseases and tumors. Unravelling the complexity of Fc-Fc γ R interactions and the requirements for activation of specific Fc γ R-mediated pathways by these therapeutic mAbs to achieve full protective activity has led to the development of Fc-optimized IgG therapeutics with improved clinical efficacy.

Signaling and Function of Anti-Tumor Antibodies

IgG antibodies are now routinely used for the treatment of a variety of human malignancies. The relative success of rituximab, the first FDA-approved mAb developed to treat cancer led to the evaluation of numerous other IgG cancer therapeutics, resulting in many mAbs 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 anti-tumor immunity. It is now clear that, independent of the cell type being targeted by the Fab domain, the Fc domain of anti-tumor therapeutic antibodies must be optimized to engage the appropriate class of Fc γ R 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 anti-tumor antibodies

The development of hybridoma technology along with identification of antigens that were specific or over-expressed on tumor, but not normal cells, enabled the exploitation of the fine specificity of antibodies to precisely target tumor cells. The first mAbs that emerged as cancer therapeutics targeting tumor-associated antigens were rituximab, an anti-CD20 mAb that targets malignant B cells in non-Hodgkin's lymphoma and chronic lymphocytic leukemia, trastuzumab, an anti-human epidermal growth receptor 2 (HER2)/neu mAb that targets HER2⁺ tumor cells in breast cancers and cetuximab, an anti-epidermal growth factor receptor (EGFR) mAb 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), and Fc-mediated pathways, such as complement-dependent cytotoxicity (CDC) 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 Fc γ R_s for depletion of target cells was found to be an absolute requirement for the *in vivo* activity of tumor-targeting mAbs, including rituximab, trastuzumab, and cetuximab.

The most striking evidence for Fc γ R-mediated mechanisms determining the anti-tumor activity of cytotoxic antibodies *in vivo* comes from work showing that anti-tumor mAbs can be therapeutically potent in wild-type mice, but not in mice lacking the FcR γ chain, which is required for the expression and signaling of all murine activating Fc γ R_s (64). Moreover, the anti-tumor effect of mAb therapy was enhanced in mice lacking the inhibitory Fc γ RIIb (47), implicating the signaling balance between activating and inhibitory Fc γ R_s in determining the potency of anti-tumor cytotoxic mAbs. This was further demonstrated through characterizing cytotoxic activities of anti-tumor mAbs of different murine IgG subclasses (2). When the variable region of the anti-gp75 TA99 mAb clone was produced with different IgG subclass scaffolds, the cytotoxic effect of the mAb was found to directly correlate with the binding affinity ratio of activating Fc γ R to inhibitory Fc γ R, or 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 γ 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 mAbs (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 γ R interactions and the impact of the binding affinity of the IgG Fc to activating Fc γ R_s has been confirmed in humans by comparing the response to these therapeutics in patients expressing high or low binding alleles of Fc γ RIIa and Fc γ RIIIa. Patients who express higher affinity Fc γ RIIa and Fc γ RIIIa alleles have enhanced response rate in 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 exploits Fc-Fc γ 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), represents an effective strategy to yield therapeutic antibodies with increased potency. Indeed, in preclinical animal studies enhanced *in vivo* activity was observed when mAbs targeting melanoma associated differentiation antigen gp75, CD19, and CD20 were Fc-engineered through point mutations to enhance affinity for activating Fc γ Rs (12, 13, 74). Similarly, a bisected, afucosylated, Fc-glycoengineered anti-EGFR mAb demonstrated enhanced ADCC activity and resulted in superior *in vivo* efficacy compared to cetuximab, a non-glycoengineered anti-EGFR mAb (75). This approach has been advanced to the clinic with the FDA approval of obinutuzumab, an afucosylated anti-CD20 mAb that, when combined with chemotherapy, extended progression-free survival of chronic lymphocytic leukemia patients compared to the wild-type IgG1 rituximab plus chemotherapy. Based on these preclinical and clinical studies, many next-generation, Fc-optimized anti-tumor mAbs have been developed and are currently being evaluated for clinical use.

While the clearance of tumor cells is readily achieved by anti-tumor targeting mAbs, evidence has accumulated suggesting that a long-term cellular memory “vaccinal” response can also be induced as a consequence of cytotoxic mAb therapy. Studies using mouse models have demonstrated that CD20 mAb treatment induces anti-tumor T-cell responses (12, 76). Similar effects in humans are supported by the long-lasting responses observed in some patients after a single course treatment with rituximab and 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) mAbs (79, 80). Recent studies have elucidated the involvement of Fc γ R pathways in the induction of anti-tumor vaccinal effects. Using human CD20 as a tumor neoantigen, anti-human CD20 mAb treatment in wild-type and in Fc γ R knock-out mouse strains revealed that both the primary clearance of the CD20-expressing tumor cells, as well as the generation of memory T-cell responses were dependent on Fc γ R interactions (12, 76). Studies in Fc γ R-humanized mice showed that the initial depletion of tumors by cytotoxic effector cells was mediated by Fc γ RIIIa on monocytes and macrophages, while the formation of a memory T-cell response was dependent on Fc γ RIIa expression on dendritic cells. Overall, this work suggested a mechanism whereby administration of an anti-CD20 mAb triggered macrophage-mediated killing of tumor cells through Fc γ RIIIa, leading to IgG immune complex formation and engagement of activating Fc γ RIIa on dendritic cells; this engagement stimulated dendritic cell activation, maturation, and cross presentation of tumor neoantigens to T cells, leading to the induction of anti-tumor T cell memory. These studies suggest novel strategies for third generation anti-tumor antibodies in which Fc engineering is designed to enhance not only cytotoxicity but also T-cell memory responses by appropriate FcR engagement.

Immunomodulatory Antibodies

Cancer therapy has been revolutionized with the development of immune checkpoint-blocking mAbs 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 use of these mAbs either as monotherapies or in combination, thus validating

the approach of targeting immune cells with specific mAbs (82-84). These mAbs act by targeting co-regulatory receptors on the surface of immune cells in order to enhance anti-tumor immune responses. Mechanistically, immunomodulatory mAbs act as agonists or antagonists to stimulate or block, respectively, a target antigen. It is now clear that many of these therapeutic mAbs depend on Fc-Fc γ R interactions to achieve full therapeutic activity. Animal studies have highlighted the importance of activating Type I Fc γ Rs for the anti-tumor activity of several immunomodulatory mAbs, such as anti-CTLA-4 (85-87), GITR (glucocorticoid-induced TNFR-related protein) (87), OX40 (88), and PD-L1 (programmed death-ligand 1)(89). These studies elucidated unexpected Fc γ R-dependent depletion of specific regulatory immune cell populations in the tumor microenvironment (TME), providing 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 mAbs act to deplete target immune cells in an activity similar to that of cytotoxic tumor-targeting mAbs described above. More specifically, the anti-tumor activity of anti-CTLA-4, GITR, and OX40 mAbs is significantly augmented by the depletion of regulatory T cells (Treg) within the TME (90). Despite the wide distribution and expression of these receptors on different T-cell compartments, mAb-mediated targeting and elimination is preferentially directed to intratumoral Treg cells. Treg cell depletion increases the ratio of Teff/Treg cells in the TME, which favors efficient tumor clearance. Simpson et al. (85) demonstrated that this specific effect of anti-CTLA-4 mAb depends on two main factors that distinguish the TME from lymphoid organs. First, Treg cells have higher expression density of surface CTLA-4 compared to effector T cells (Teff) and CTLA-4 expression level is elevated in the tumors compared to lymphoid organs. Thus, higher receptor expression density preferentially targets Treg for ADCC. Secondly, the depletion of the Tregs by CTLA-4 mAbs is dependent on Fc γ RIV-expressing macrophages which are more abundant and express higher levels of Fc γ 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 high A/I ratio in order to mediate the therapeutic activity of CTLA-4 mAbs. Similar requirements for receptor over-expression by intratumoral Treg cells and the presence of myeloid cells expressing activating Fc γ Rs in the TME have been characterized as a general mechanism for additional immunomodulatory mAbs. The therapeutic activity of mAbs targeting the activating receptors GITR and OX40 depends on their ability to modulate the TME by depleting intratumoral Treg cells. An alternative mechanism to immunomodulate the TME has been described for anti-PD-L1 mAbs, which target the ligand for PD-1 (89). PD-L1 mAbs were found to have superior anti-tumor activity when engaged to activating Fc γ Rs to deplete intratumoral myeloid cells, and thereby modulating myeloid cell populations in the TME. This Fc γ R-dependent mechanism synergized with the Fc γ R-independent blocking of the inhibitory PD-1/PD-L1 axis for optimal therapeutic efficacy.

Evidence for Treg depletion in patients was recently demonstrated in small groups of melanoma patients treated with ipilimumab, the FDA-approved anti-CTLA4 IgG1 mAb. In these patients, a higher baseline frequency of peripheral non-classical monocytes and tumor-associated macrophages that expressed Fc γ RIIIa was associated with treatment responsiveness. Furthermore, these non-classical monocytes were shown to mediate Treg

cell depletion by ipilimumab *ex vivo* (91). A second human anti-CTLA-4 mAb that has been used clinically is tremelimumab, which is an IgG2, and therefore has significantly reduced Fc γ RIIIa 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 treatment exhibits some therapeutic activity in combinational trials assessing tremelimumab in combination with other anticancer drugs (92). This activity may represent the multiple mechanisms that can be induced by CTLA-4 mAbs in humans. Further studies on these therapeutic mAbs are needed to fully exploit their mechanism of action in patients. Interestingly, PD-L1 mAbs that demonstrate clinical activity are either human IgG4, which have low Fc γ R binding and ADCC activity, or an Fc-null version mutated to abrogate Fc γ R binding. One PD-L1 mAb 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 Fc γ Rs, as has been observed in preclinical models using other anti-PD-L1 antibodies.

Recent studies revealed an unexpected requirement for engagement of the inhibitory Fc γ RIIb for optimal agonistic activity of mAbs targeting members of the TNFR superfamily, including CD40, DR5, CD30, and Fas (93-96). Contrary to antitumor cytotoxic and immunomodulatory mAbs that require activating Fc γ R engagement and signaling to mediate specific elimination of target cell populations, engagement of the inhibitory Fc γ RIIb by anti-TNFR mAbs does not require Fc γ R intracellular signaling to mediate IgG agonistic activity. Fc γ RIIb potentiates the activity of agonistic mAbs by solely providing a scaffold, in *trans*, for antibody crosslinking, enhancing thereby target receptor clustering on the cell surface. Fc γ RIIb engagement is mainly required for the activity of mAbs targeting receptors that depend on multimerization for their activation, such as members of the TNFR family. Whereas soluble, non-crosslinked mAbs fail to mimic the trimeric TNFR-TNF complex needed for activation, Fc γ RIIb-crosslinked mAbs have the capacity to confer potent agonistic activity *in vivo*. A similar requirement for Fc γ RIIb engagement was observed for the agonistic activity of mAbs targeting a member of the class A scavenger receptor family (MARCO; macrophage receptor with collagenous structure) (97). Anti-MARCO mAbs mediate reprogramming of MARCO-expressing tumor associated macrophages (TAMs) from a pro-tumoral, immunosuppressive M2 phenotype to an M1-like one, modulating the TME into more inflammatory and anti-tumoral state. Similar to anti-TNFR mAbs, Fc γ RIIb 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 γ RIIb engagement, Fc γ RIIb-targeted Fc engineering could enhance their *in vivo* antitumor activity. CD40 is perhaps the most studied therapeutic target for the Fc γ R-dependent crosslinking pathway. Agonistic anti-CD40 mAbs represent physiologic adjuvants, which 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 of tumors in multiple models (98). Indeed, studies in Fc γ R-humanized mice revealed that agonistic anti-CD40 antibodies optimized for enhanced human Fc γ RIIb binding displayed a remarkable (>10-fold) improvement in their

immunostimulatory and anti-tumor 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 γ RIIb-targeted Fc engineering (94, 95). Several anti-human CD40 antibodies have been developed, and among them, CP-870,893 represents the most potent agonistic anti-human CD40 mAb 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 γ 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 γ RIIb (102). Indeed, subclass switching of CP-870,893 to human IgG1 significantly improved its *in vivo* activity, due to the increased affinity of human IgG1 for Fc γ RIIb. Likewise, Fc domain engineering to selectively enhance affinity of the IgG1 Fc for Fc γ RIIb further improved the *in vivo* antitumor activity of CP-870,893, indicating that Fc variants specifically enhanced for Fc γ RIIb binding may be the optimal IgG framework for agonistic anti-CD40 mAbs. This work further highlights the utility of animal models humanized for both the target antigen and Fc γ 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 Ig 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 γ R-mediated effector functions, genetic variation in Fc γ Rs can underlie differences in susceptibility to infectious diseases. For example, the low affinity variant of Fc γ RIIa (R131) confers increased susceptibility to severe bacterial infections and sepsis, likely due to 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 γ RIIa polymorphism is associated with asymptomatic infection and improved clinical outcomes (105, 106). IgG-mediated immunity against infection occurs through numerous mechanisms involving Fc γ 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 actually play a role in *in vivo* protection cannot be reliably predicted from *in vitro* experiments. Here, we will review the mechanisms of IgG-mediated protection *in vivo* using studies of immunity to influenza viruses and to HIV as model systems.

Influenza

Neutralizing antibodies (nAb) against influenza viruses bind the HA, which is responsible for mediating viral entry into host cells. Anti-HA nAbs 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 nAbs (those that prevent attachment or fusion) can be distinguished *in vitro* using the classic hemagglutination inhibition (HI) assay, which detects nAbs that act by preventing attachment of HA to sialic acids, or, in cell-based microneutralization assays, which detect nAbs that act by either mechanism. NAbs 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 nAbs occurs for three reasons: 1) 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, 2) the rapid kinetics of influenza virus replication results in efficient selection of escape variants when selective pressure of a nAb is applied, and 3) the ability of antigenic sites in the HA to accommodate tremendous structural diversity means that the virus can nearly always escape nAbs that bind to those regions. Because the nAbs that prevent viral attachment readily select for escape variants, they are, by nature, strain-specific (113). *In vivo*, nAbs that prevent attachment are generally protective at very low concentrations and those that have been studied protect without a requirement for Fc γ R engagement (35).

In contrast to nAbs that prevent viral attachment, those 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 nAbs 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 nAbs that prevent viral attachment (107). New influenza vaccine candidates designed to elicit broadly neutralizing antibodies, often aim to elicit these fusion-inhibiting nAbs because of the breadth of protection that they can mediate *in vivo*. Several fusion-inhibiting nAbs have been studied for Fc γ R requirements and were shown to protect without a requirement for Fc γ R engagement at relatively high concentrations, but activating Type I Fc γ Rs were required for their activity at lower concentrations (114, 115). Thus, potency of broadly-neutralizing nAbs is dependent on Type I Fc γ Rs *in vivo*.

In contrast to nAbs, which can confer sterilizing immunity against influenza viruses, non-neutralizing 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 nAbs, non-neutralizing antibodies can be broadly reactive and broadly protective *in vivo*; they are generally of lower protective potency than the nAbs that prevent viral attachment and several broadly reactive anti-HA or neuraminidase mAbs have been shown to be dependent on Type I Fc γ Rs for potent *in vivo* protection (114). The cumulative

data support a model where strain-specific nAbs do not require recruitment of Fc γ R-mediated effector functions, while broadly protective antibodies depend on activating Type I Fc γ R interactions for potent activity *in vivo*.

Interestingly, in addition to the varying requirements of different anti-influenza IgGs for Fc γ 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 nAbs that have been studied that prevent viral attachment do not readily engage Fc γ RIIIa without Fc engineering to increase Fc-Fc γ 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 γ Rs. In contrast however, nAbs and mAbs with broad protective activity *in vivo* do generally require Fc γ R engagement and their Fcs are able to engage Fc γ RIIIa to mediate ADCC *in vitro* (114, 115). Determinants of Fc γ R engagement by bound IgGs are not fully understood but they include binding density, which is dictated by nature and expression of antigen, and may also be impacted 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 (1 Fc domain per 1 bound Fab domain). In contrast, higher affinity IgGs are more likely to form bivalent interactions with antigen (1 Fc domain per 2 bound Fab domains), limiting the potential for dense IgG binding (117). ADCC mediated by broadly reactive anti-HA mAbs may also be supported through increased affinity between target and effector cell due to broadly neutralizing mAb Fc-Fc γ RIIIa engagement in conjunction with binding by the viral HA to sialic acids on the effector cell (118). Yet another mechanism that may impact the ability of a bound IgG to engage Fc γ 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 γ R interactions. A correlate to this is that IgG constant domains can impact fine specificity of the variable domain; this has been demonstrated in studies showing that identical variable domains in IgGs of distinct subclass can have different binding specificities (122).

An area for further investigation in Fc γ R signaling and influenza biology is the extent to which effector functions mediated by activating Type I Fc γ 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 γ R-independent selective pressure by nAbs 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 impact the antigenic landscape of circulating influenza viruses. Vaccines that elicit broadly reactive HA or neuraminidase IgGs will likely protect *in vivo* largely through Fc γ 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-1 are specific for the major envelope (Env) glycoprotein 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 and 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 could be achieved through passive administration of broadly neutralizing anti-Env mAbs 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 mAb which disrupts interaction with CD4 can suppress viremia in chronically infected HIV-1 patients (127).

Mechanistic studies have demonstrated increased *in vivo* activity of anti-Env nAbs with capacity for enhanced binding to activating Type I Fc γ R_s in preventing HIV-1 entry, suggesting a role for activating Fc γ R_s in *in vivo* neutralization (33). Similarly, activating Fc γ R_s have been shown to mediate *in vivo* clearance of HIV-infected cells, further contributing to the antiviral activity of anti-Env nAbs (128). Further assessment of the protective activity of Fc variants of anti-Env mAbs with differential Fc γ R binding capacities in post-exposure prophylaxis and therapy models of HIV-1 infection in humanized mice revealed a requirement for activating Fc γ R engagement for optimal therapeutic activity of anti-Env nAbs (33, 129). These findings are consistent with previous observations using a non-human primate pre-exposure prophylaxis model, in which Fc domain variants with minimal capacity for Fc γ R 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 Fc γ R_s are required for this activity *in vivo*. Given the contribution of Fc γ R-mediated pathways in the *in vivo* antiviral activity of anti-Env nAbs, Fc domain engineering for selective engagement of activating Fc γ R_s is expected to lead to the development of anti-Env nAbs with improved therapeutic efficacy.

Other infectious diseases

Studies support an important role for Fc γ R signaling in protection against a number of pathogens in addition to influenza viruses and HIV. For example, passive transfer of an anti-RSV mAb, palivizumab, with enhanced affinity for the activating Fc γ R_{IIIa} by virtue of modification with afucosylated Fc glycoforms, reduced RSV lung titers *in vivo* compared with the fucosylated version; abrogation of palivizumab-Fc γ R binding further reduced *in vivo* protection, demonstrating that Fc γ R-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 Fc γ R_s. Fc γ R_{IIIa} was shown to be required for mAb-mediated protection against *S. pneumoniae* and numerous experiments

have shown that subclass determines protective activity of IgGs against *S. aureus* and *C. neoformans*, with affinity for activating Fc γ Rs correlating with improved protection (reviewed in (131)). Based on these studies, mAbs engineered for selectively enhanced binding to activating Fc γ Rs have been developed as passive transfer therapeutics; the *in vivo* activity of a mAb against the lethal toxin of *B. anthracis*, for example, was improved through Fc optimization that enhanced binding to activating human Fc γ Rs (34) and, as described above, similarly engineered mAbs have shown increased potency in suppression of HIV rebound (33, 129). In addition to activating Fc γ Rs, signaling through the inhibitory Fc γ RIIb can also affect susceptibility to infections since it offsets activating Fc γ R effects. For example, *Fcgr2b*^{-/-} mice exhibit improved bacterial clearance in models of pneumococcal peritonitis through improved macrophage effector function (132, 133), whereas overexpression of Fc γ RIIb is associated with increased mortality upon challenge with *Streptococcus pneumoniae* (132, 133). Collectively, these findings demonstrate the importance of IgG-Fc γ 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 γ 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; while 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 anti-dengue 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 γ 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 non-neutralizing, 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 γ 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 pre-existing, non-neutralizing anti-dengue IgGs develop severe disease (146). Analysis of IgGs from patients with mild or severe dengue disease showed that patients with severe disease have Fc structures on anti-dengue IgGs that are selectively enhanced for Fc γ RIIIa binding (TTW and JVR, unpublished observation). This suggests that production of IgGs with higher affinity for Fc γ 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 due 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 a RSV vaccine trial in 1968 in which children who received the experimental vaccine were >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 virus that elicited a large proportion of non-neutralizing antibodies that are thought to have formed insoluble immune complexes with viral proteins which 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 1957 or 2009 pandemic influenza infections 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 γ 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, non-neutralizing IgGs which may mediate pathogen uptake by Fc γ R-expressing cells or, in rare circumstances, may form insoluble immune complexes that enhance inflammation and disease due to complement-mediated inflammation, Type I Fc γ R-mediated inflammation or direct cytotoxicity.

Conclusions

IgG antibodies represent 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 Fc γ Rs. In addition to the direct role that IgG antibodies play in protection against malignancies and infections, IgG-mediated signaling also has a key role in shaping adaptive immune responses. This is because antigen is often 'seen' *in vivo* as IgG immune complexes since IgG antibodies that were pre-existing 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 previously in this chapter. Crosslinking of activating Type I Fc γ Rs 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 Fc γ RIIIa 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 Fc γ R, CD23, on B cells to trigger upregulation of the B cell inhibitory Fc γ RIIb. Increased Fc γ RIIb 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 Fc γ R in mediating effector activities and modulating adaptive immune responses. Fc domain engineering of therapeutic antibodies to engage particular classes of Fc γ R substantially improved their clinical efficacy. Given the contribution of Fc-Fc γ R interactions to the modulation of adaptive immune responses, specific activation of Fc γ R-mediated pathways during vaccination could also lead to the generation of robust and sustained adaptive immune responses, with broad and potent protective activity.

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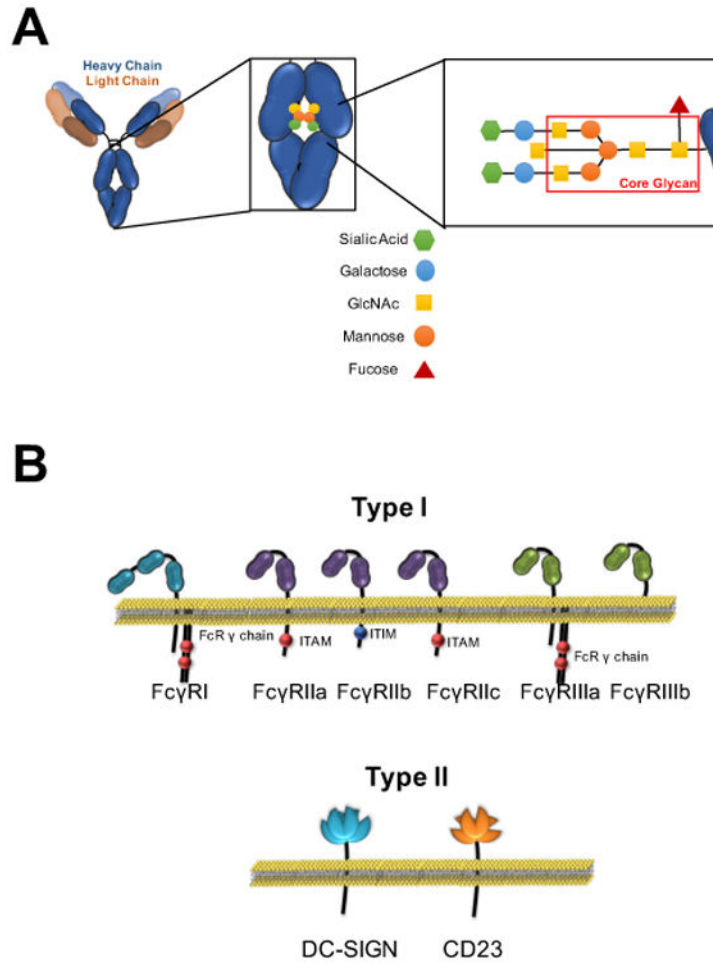


Figure 1. Overview of the structural characteristics of IgG (A) and FcγRs (B)
 IgG antibodies comprise two identical heavy and light chains linked by inter-chain disulfide bonds. At the core of the Fc domain, an *N*-linked glycan structure is conjugated at a highly-conserved site at the CH2 domain (Asn297 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 (highlighted in the red box), which in turn determines the binding affinity of the Fc domain for Type I and Type II FcγRs. Despite their common property to interact 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.

Table 1
Expression pattern of Type I and Type II FcγRs among the various leukocyte types

	Type I					Type II		
	FcγRI	FcγRIIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIb	DC-SIGN	CD23
B cells	-	-	+	-	-	-	-	+
T cells	-	-	-	-	-	-	-	#
NK cells	-	-	- ^Δ	+ ^Δ	+	-	-	-
Monocytes	+	+	+	-	+/-	-	+/-	#
Neutrophils	#	+	+	-	-	+	-	#
Eosinophils	#	+	+	-	-	#	-	#
Macrophages	+/-	+	+	-	+/-	-	+/-	#
Dendritic Cells	#	+	+	-	#	-	+	-

⁺ constitutive expression;

⁻ no expression;

[#] inducible expression;

^{+/-} expression restricted to certain subsets;

^Δ expression depends on FcγR genetic variation.

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

Cell type	Role of FcγR	Short-term Effects	Long-term Effects
Granulocytes	+ FcγRIIIa FcγRIIIb*	Degranulation ROI production Phagocytosis	Release of pro-inflammatory molecules Cell survival and motility Impact on other myeloid leukocytes
Monocytes	FcγRIIa FcγRIIIa#	Phagocytosis Cytokine & chemokine expression	Monocyte recruitment and differentiation, cell survival, stimulation of pro-inflammatory pathways
<i>Myeloid</i> Macrophages	FcγRIIa FcRIIIa DC-SIGN	Phagocytosis Cytokine & chemokine expression	Influence 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	Cell maturation, upregulation of co-stimulatory molecules	Enhanced antigen processing and presentation, impact on T-cell responses
B cells	CD23 FcγRIIb	B cell selection	Generation of high-affinity IgG responses
<i>Lymphoid</i> Plasma cells	FcγRIIb	B cell survival	Regulation of plasma cell apoptosis and control of IgG production
NK cells	FcγRIIIa	Cell activation Degranulation	Cytotoxicity, cell survival, impact on other effector leukocytes, generation of immune complexes

+ activates downstream effects;

- inhibits downstream effects

* function unknown, possible activating;

expressed on certain subsets.