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Fc γ Receptor Pathways during Active and Passive Immunization

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Summary

IgG antibodies are actively produced in response to antigenic challenge or passively administered as an effective form of immunotherapy to confer immunity against foreign antigens. Their protective activity is mediated through their bifunctional nature: a variable Fab domain mediates antigen binding specificity, whereas the constant Fc domain engages Fc γ receptors (Fc γ Rs) expressed on the surface of leukocytes to mediate effector functions. While traditionally considered the invariant domain of an IgG molecule, the Fc domain displays remarkable structural heterogeneity determined primarily by differences in the amino acid sequence of the various IgG subclasses and by the composition of the complex, Fc-associated bi-antennary *N*-linked glycan. These structural determinants regulate the conformational flexibility of the IgG Fc domain and affect its capacity to interact with distinct types of Fc γ Rs (type I or type II Fc γ Rs). Fc γ R engagement activates diverse downstream immunomodulatory pathways with pleiotropic functional consequences including cytotoxicity and phagocytosis of IgG-coated targets, differentiation and activation of antigen presenting cells, modulation of T cell activation, plasma cell survival and regulation of antibody responses. These functions highlight the importance of Fc γ R-mediated pathways in the modulation of adaptive immune responses and suggest a central role for IgG-Fc γ R interactions during active and passive immunization.

Keywords

Fc receptors; Antibodies; Vaccination; Immunotherapies; Inflammation

Introduction

Antibodies are key components of our immune system that link the two main branches of immunity: innate and adaptive. These Y-shaped molecules are selected and produced by cells of the adaptive immune system (B cells), whereas their subsequent binding to innate leukocytes triggers a range of effector functions that provide effective host protection. Recognition and binding of antibodies to the surface of the leukocytes is mediated through their Fc domains interacting with specialized receptors, Fc receptors, expressed by several types of circulating and tissue-resident leukocytes. Contrary to the antigen-binding Fab domain that exhibits astonishing variability due to the almost unlimited diversification of its

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variable regions (VH and VL), the Fc domain is relatively constant, allowing thereby its recognition by innate receptors, like Fc receptors. Despite its invariant nature, the Fc domain exhibits some degree of heterogeneity, as a result of differences in the primary amino acid backbone sequence among the various IgG subclasses as well as in the structure and composition of the Fc-associated glycan (1, 2). Recent crystallographic studies revealed that the IgG Fc domain exhibits two main conformational states ('open' or 'closed') that are physiologically regulated by the Fc-associated glycan structure and consequently affect Fc γ R binding (3).

Fc γ Rs are broadly categorized into type I or type II based on their capacity to interact with the two conformational states of the IgG Fc domain (4, 5). Type I Fc γ Rs include the canonical Fc γ Rs and can be engaged by the IgG Fc only in the 'open', but not in the 'closed' conformation. In contrast, type II Fc γ Rs, which include C-type lectin receptors, like CD23 and DC-SIGN exhibit minimal affinity for the 'open' IgG Fc conformation, binding preferentially IgG Fcs at the 'closed' conformation. The dynamic flexibility of the IgG Fc domain and its capacity to interact with the different Fc γ R types greatly influences the *in vivo* effector functions of antibodies. Engagement of Fc γ Rs expressed on the surface of effector leukocytes initiate a number of pro-inflammatory, anti-inflammatory and immunomodulatory functions with pleiotropic effects that contribute to the *in vivo* protective activity of antibodies, and modulate the host adaptive immune responses. In this review, we will focus on the mechanisms that regulate IgG binding to Fc γ Rs and provide an overview of the activity and function of Fc γ Rs. We will highlight the mechanisms by which Fc γ R engagement by passively-administered antibodies provide *in vivo* protection and discuss the evidence on the role of Fc γ R-mediated pathways in driving humoral and cellular immune responses upon active immunization.

IgG Fc domain structural and functional heterogeneity

IgG is the most abundant immunoglobulin class in serum, constituting over 75% of circulating immunoglobulin. It is the main immunoglobulin class that is produced during an immune response to provide efficient protection against foreign antigens. Common to the other immunoglobulin classes, IgG molecules are comprised of two identical heavy (H) and light (L) chain domains that are characterized by genetically variable (V) or constant (C) regions. Each domain is folded into a globular immunoglobulin motif (four for the heavy chain and two for the light chain), and the IgG molecule is divided into two main domains linked by a hinge region that ensures pairing of the two heavy chains through disulphide bonding. These domains include the Fab (fragment, antibody binding) domain, which includes the variable regions that mediate antigen binding), and the Fc (fragment, crystallizable) domain that primarily serves as the binding site for Fc γ Rs, FcRn, complement and other receptors or proteins (e.g. TRIM, Streptococcal protein A or G)(6).

Contrary to the sequence variability of the Fab domain, the Fc domain is comprised of the constant domains (CH2 and CH3) of the two heavy chains and adopts a horseshoe-like conformation that is formed by the tight association of the two CH3 domains at the C-terminal proximal region of the IgG, while the CH2 domains remain further apart (7). This characteristic conformation is primarily achieved by the presence of a central N-linked

glycan structure conjugated to the amino acid backbone of the CH2 domain at position Asn297 of both heavy chains (8, 9). This carbohydrate structure lies within the hydrophobic cleft of the Fc domain formed by the two CH2 domains and dynamically regulates the Fc domain conformational structure and thereby its capacity to interact with type I or type II Fc γ Rs (3, 5, 10). The presence of the Fc-associated glycan is required to maintain the Fc domain in a structural conformation that would permit its interaction with Fc γ Rs (10). Indeed, loss of this glycan either through enzymatic removal or mutation of the Asn297 residue greatly impacts the capacity of the Fc domain to interact with all classes of type I and type II Fc γ Rs (10–13).

Like any other process of the immune system, IgG binding to the different Fc γ R types is tightly regulated and several layers of control exist that dynamically regulate this process to eliminate any potential for uncontrolled or inappropriate inflammation. Differences in the amino acid backbone between the various IgG subclasses (IgG1, IgG2, IgG3, IgG4 in humans) and the Gm allotypes readily affect the capacity of the hinge-proximal region of the Fc domain to interact with type I FcRs (14, 15). Likewise, the impact of specific mutations at the CH2 domain, particularly within the interface that participates in the interaction with type I Fc γ R, has been assessed in several studies and a number of protein sequence variants have been identified that specifically alter the affinity of the IgG Fc domain for particular classes of type I Fc γ Rs (14, 16–18). These studies highlight the importance of the primary amino acid backbone sequence of the IgG Fc domain in regulating Fc-Fc γ R interactions and provided the basis for the generation and evaluation of Fc domain variants engineered for improved Fc effector activity through enhanced capacity for Fc γ R engagement (19–22).

Apart from the amino acid sequence of the IgG Fc domain, engagement of type I and type II Fc γ Rs is also regulated by the structure and composition of the Fc-associated glycan (4). An ever-increasing body of evidence strongly suggests a crucial role for the Fc-associated glycan in the modulation of the IgG Fc effector activity (8, 10). The complex, biantennary Fc glycan consists of a core heptasaccharide structure attached to Asn297; a highly conserved N-linked glycosylation site among the different IgG subclasses and among many mammalian species (1). Analysis of the Fc glycan composition revealed substantial heterogeneity, due to the addition of fucose, galactose, N-acetylglucosamine and sialic acid residues to the core glycan structure. While the presence of the core glycan structure is necessary to preserve the CH2 domains of the two heavy chains at a conformation permissive for Fc-Fc γ R interactions (8, 11), it is the structure and composition of the glycan that finely tunes the affinity of the Fc domain for binding to the different classes of type I and type II Fc γ Rs (3, 4, 10). For example, the presence of a branching fucose has been shown to reduce affinity specifically for the activating type I Fc γ R, Fc γ RIIIa, without any impact on the capacity of the Fc domain to interact with other classes of type I Fc γ Rs (23, 24). Indeed, afucosylated IgG glycovariants exhibit improved *in vivo* Fc effector activity over their fucosylated counterparts and the approach of Fc glycoengineering has been successfully used to generate antibodies with improved *in vivo* efficacy (25–28). Likewise, the presence of terminal sialic acid residues abrogates binding to type I Fc γ Rs and enables preferential engagement of type II Fc γ Rs (29, 30). Molecular modeling and analysis of the crystal structure of sialylated IgG Fc revealed that sialylation induces a conformational change of the CH2 domains that affects type I and type II Fc γ R binding (3, 5). At the

conformational state induced by the sialylated glycan, the type II Fc γ R binding site, located at the CH2-CH3 interface is exposed enabling the interaction with type II Fc γ Rs, whereas the hinge-proximal region of the CH2 domain that mediates type I Fc γ R binding becomes inaccessible (3–5). It is therefore well-established that the Fc glycan structure plays a central role in regulating the dynamic flexibility of the IgG Fc domain and consequently its capacity to interact with type I or type II Fc γ Rs, by adopting two mutually exclusive conformations: an ‘open’ that enables for type I, but not type II Fc γ R binding, and a ‘closed’ that is induced upon sialylation and preferentially engages type II, but not type I Fc γ Rs (4).

Engagement of the different Fc γ R types induces divergent immunomodulatory responses that can greatly affect the outcome of IgG-mediated inflammation and immune responses. Given the significance of the effector responses induced upon Fc γ R engagement, it is anticipated that several regulatory mechanisms have evolved to control IgG Fc glycan composition. Although the precise mechanisms are still poorly characterized, there is substantial evidence suggestive of the existence of homeostatic processes that regulate Fc glycan composition, and more specifically the activity of ST6Gal1, the glycosyltransferase responsible for terminal Fc glycan sialylation. For example, Fc glycan analysis composition upon vaccination revealed specific modulation in the abundance of antigen-specific IgG with sialylated Fc domains, which potentially has immunomodulatory consequences for the subsequent immune response (31, 32). Likewise, analysis of the levels of sialylated IgG Fc in patients with autoimmune pathologies, like rheumatoid arthritis and Wegener’s granulomatosis revealed an association with clinical disease severity (33–36). In particular, higher levels of sialylated Fc in autoantibodies were observed during periods of clinical remission, whereas disease relapses were commonly associated with a significant decrease in IgG Fc sialylation (33, 34). These observations highlight the importance of the Fc glycosylation status in regulating IgG Fc effector activity and suggest the presence of homeostatic mechanisms that dynamically control IgG Fc glycan structure and composition.

Fc γ R function and activity

Type I Fc γ R Family

Based on their capacity to interact with the two main conformational states of the IgG Fc domain, Fc γ Rs are broadly divided into type I and type II. Type I Fc γ Rs is a group of immunoreceptors that are structurally and functionally related and belong to the immunoglobulin (Ig) superfamily (37, 38). Their extracellular, IgG-binding domain is comprised of two (or three for Fc γ RI) Ig domains that mediate interactions with the IgG through the hinge proximal region of the CH2 domain in a 1:1 complex (14, 15). They share highly conserved intracellular signaling components that mediate receptor signaling upon crosslinking by IgG-antigen complexes (39). In humans, eight different genes, each with multiple transcriptional isoforms, encode the various classes of type I Fc γ Rs (Fc γ RI, Fc γ RIIa/b/c, and Fc γ RIIIa/b)(Figure 1). Most of these genes are mapped at a common Fc γ R locus (located at 1q23) that originated through sequential non-homologous recombination events of the ancestral Fc γ R locus that is highly conserved among mammalian species and can be traced back early in evolutionary history (40, 41). Despite the sequence similarity shared among the different classes of type I Fc γ Rs, they present

distinct structural and functional characteristics (37)(see also Hargraves et al. elsewhere in this volume for an analysis of the Fc γ R locus).

Fc γ RI is a high affinity Fc γ R that is capable of binding monomeric IgG (K_D 10^{-9} - 10^{-10} for human IgG1). Its increased affinity for IgG is due to its unique structure; the presence of a third (D3), extracellular Ig domain (compared to Fc γ RII or Fc γ RIII) acts as a spacer for the Fab domain, stabilizing thereby IgG-Fc γ Rs interactions (42). Fc γ RI is constitutively expressed predominantly by monocytes and macrophages and can be induced in other myeloid leukocyte types, like neutrophils, primarily by interferon- γ and to a lesser extent by granulocyte-colony stimulating factor (G-CSF), interferon- α and interleukin-12 (43, 44). The ligand-binding, α chain of Fc γ RI associates with the FcR γ -chain homodimer; a process required for cell surface expression and signal transduction (45–47). Although three genes encoding Fc γ RI have been identified (*FCGR1A*, *FCGR1B*, *FCGR1C*), only *FCGR1A* expresses the functional Fc γ RI, whereas *FCGR1B/C* represent duplicated pseudogenes of *FCGR1A* that may express truncated or soluble forms of Fc γ RI due to the presence of a premature stop codon within the extracellular region of Fc γ RI (48, 49).

Contrary to Fc γ RI, the other type I Fc γ R classes, Fc γ RII and Fc γ RIII exhibit low affinity for IgG (K_D for hIgG1: 10^{-5} - 10^{-7}). While they are incapable of binding monomeric IgG, IgG-antigen complexes (immune complexes) can sufficiently engage and crosslink low affinity Fc γ Rs through high avidity, multimeric interactions (50). There are three different Fc γ RII and two Fc γ RIII classes; each with unique structural and functional properties. All the different classes of Fc γ RII share a characteristic structure that includes a functional signaling motif within the cytoplasmic region of the receptor α chain and can therefore transduce intracellular signals following receptor crosslinking without the need for accessory signaling subunits, like the FcR γ -chain. Fc γ RIIa, Fc γ RIIb, and Fc γ RIIc are encoded by *FCGR2A*, *FCGR2B*, and *FCGR2C*, respectively. Fc γ RIIa encompasses an immunoreceptor tyrosine-based activation motif (ITAM) at its cytoplasmic tail and is widely expressed by several leukocyte types of myeloid origin, including granulocytes, monocytes, macrophages, dendritic cells, and platelets (37). By contrast, Fc γ RIIb is the sole inhibitory Fc γ R and its activity is mediated through an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) (6). Fc γ RIIb is expressed as two splicing variants (Fc γ RIIb1 and Fc γ RIIb2) that differ in their capacity to become internalized following receptor crosslinking (51, 52). Fc γ RIIb1, the full-length transcript variant, includes a signal sequence that inhibits receptor internalization and is exclusively expressed by cells of the lymphoid lineage (B cells), whereas the expression of Fc γ RIIb2, the variant that is permissive for internalization, is restricted to myeloid cells, like monocytes, macrophages and dendritic cells (52). Fc γ RIIc represents essentially a chimeric receptor that is comprised of the extracellular domain of Fc γ RIIb and the intracellular of Fc γ RIIa. Indeed, *FCGR2C*, the gene coding for Fc γ RIIc is the result of a non-homologous recombination between the *FCGR2A* and *FCGR2B* genes and its expression is restricted to NK cells (37, 40, 41). However, Fc γ RIIc expression at protein level is limited to 10–25% of the population due to a common SNP (Q57X) at exon 3 that generates a stop codon (53, 54).

The other low affinity Fc γ R class, Fc γ RIII is encoded by the *FCGR3A* and *FCGR3B* genes. While both genes share very high levels of sequence homology, their products exhibit

remarkable structural differences that influence their activity and function (41). Fc γ RIIIa is a transmembrane protein that associates with the FcR γ -chain for expression and signaling, similar to Fc γ RI (46, 55, 56). In contrast, Fc γ RIIIb is post-translationally processed as a glycosylphosphatidylinositol (GPI)-anchored protein and therefore lacks transmembrane and intracellular domains (56). It doesn't require the FcR γ -chain for expression; however, it synergistically acts with the activating receptor Fc γ RIIa to mediate cellular responses and it has been shown to associate with the FcR γ -chain (57, 58). The structural differences between Fc γ RIIIa and Fc γ RIIIb are attributed to a point mutation (Phe203Ser) in the membrane proximal region of the extracellular domain of Fc γ RIIIb that created a GPI-anchor signal sequence. Additionally, Fc γ RIIIa and Fc γ RIIIb exhibit distinct expression patterns (58). While Fc γ RIIIb expression is restricted to neutrophils, Fc γ RIIIa is widely expressed by several leukocyte cell types, including macrophages, NK cells and monocyte subsets (inflammatory monocytes, CD14^{low})(4, 37).

From a functional point of view, type I Fc γ Rs are classified into activating or inhibitory based on their capacity to transduce immunostimulatory or immunosuppressive signals following receptor crosslinking (12, 39). Both activating and inhibitory Fc γ Rs are expressed by most leukocyte types. Notable exceptions include NK cells, which express only activating Fc γ Rs (Fc γ RIIIa), and B cells, which only express the inhibitory Fc γ RIIb. Since most effector leukocytes co-express both activating and inhibitory Fc γ Rs, the outcome of IgG-mediated inflammation and immunity is largely determined by balancing activating or inhibitory signals transduced by activating or inhibitory Fc γ Rs, respectively (12). Activating Fc γ Rs, which include Fc γ RI, Fc γ RIIa/c and Fc γ RIIIa are characterized by the presence of intracellular ITAMs either in the receptor α chain (in the case of Fc γ RIIa/c) or in the associated FcR γ - chain (Fc γ RI and Fc γ RIIIa)(4, 59, 60). Despite their structural differences, all ITAM-bearing Fc receptors follow an identical pattern of signal transduction following crosslinking by immune complexes (Figure 2). In particular, binding of multimeric IgG immune complexes to activating Fc γ Rs causes receptor clustering and aggregation that in turn results in the phosphorylation of the ITAM domains (45, 58, 59, 61). Phosphorylation of the ITAM tyrosine residues induces the subsequent activation of a number of cytoplasmic tyrosine kinases, firstly those of the Src (Lyn, Lck, Hck and Fgr) and then of the Syk family (56, 59, 61–65). Furthermore, receptor aggregation and ITAM phosphorylation leads to the activation of ζ chain subunits, ZAP-70 and FAK that subsequently regulate actin polymerization, phagocytosis and receptor internalization (59, 61, 66). Src and Syk kinase family activation sequentially induces the activation of the PI3K-PKC pathway, leading to Ca²⁺ mobilization and cellular activation (67–70). In addition to these early signaling events, several other late signaling pathways become activated and include the MEK and MAP family kinases and the Ras pathway, resulting in the transcription of pro-inflammatory cytokines and chemokines with effects on cellular survival and differentiation (68, 71, 72). Although it is generally anticipated that any intracellular signals transduced upon receptor crosslinking ultimately lead to cellular activation and receptor internalization, the precise biological responses following activating Fc γ R engagement greatly vary between different leukocyte cell types. Such responses include antibody-dependent cellular cytotoxicity (ADCC) or, phagocytosis (ADCP), release

of cytokines and chemokines, leukocyte differentiation and survival, as well as modulation of T and B cell responses (4, 39).

The signaling activity of the activating Fc γ R_s is counterbalanced by the inhibitory type I Fc γ R, Fc γ R_{IIb}. Its importance in regulating Fc γ R-mediated pathways is reflected by the association of several autoimmune and chronic inflammatory pathologies with functional polymorphisms within the promoter region or the coding sequence of the *FCGR2B* gene that affect receptor expression or activity, respectively (37). Several studies have elucidated the molecular mechanisms by which Fc γ R_{IIb} mediate immunosuppressive signals. Critical for Fc γ R_{IIb} activity is the recruitment of SHIP phosphates to its ITIM domain following receptor crosslinking and phosphorylation by Src family kinases (73–75). Recruited phosphatases, like SHIP and SHP2 promote the hydrolysis of phosphatidylinositol 3,4,5-triphosphate to phosphatidylinositol 4,5-biphosphate that in turn inhibit the recruitment and activation of PLC γ and BTK (73, 76, 77). These intracellular effects balance any signals mediated by activating Fc γ R_s, effectively limiting cellular activation and effector responses. Alternatively, in the absence of activating Fc γ R_s, as in the case of B cells that express only the inhibitory Fc γ R_s, Fc γ R_{IIb} engagement regulates cellular survival and antibody production (78).

Lastly, in contrast to activating or inhibitory type I Fc γ R_s, Fc γ R_{IIIb} lacks any intracellular signaling motifs and is therefore incapable of inducing or inhibiting cellular activation. Despite the absence of functional signaling domains, Fc γ R_{IIIb} has been shown to transduce activation signals following receptor crosslinking, mainly by associating and acting synergistically with other receptors, such as Fc γ R_{IIa} and complement receptors (57). In addition, due to its GPI-anchor, Fc γ R_{IIIb} is preferentially localized in lipid raft microdomains that are rich in membrane-associated kinases, like Syk and might utilize such signal transduction machinery to initiate intrinsic signals following receptor engagement (57, 58, 79).

Type II Fc γ R Family

Type II Fc γ R_s belong to the C-type lectin family of receptors and are capable of binding to the IgG Fc domain in the ‘closed’ conformation at the CH2-CH3 interface in a 2:1 complex (receptor:IgG)(3–5). At this conformational state, binding of type I Fc γ R_s is inhibited, since the hinge proximal region of the CH2 domain, where type I Fc γ R_s normally bind, is not accessible (3). So far, two receptors have been shown to mediate binding of the ‘closed’ conformation IgG Fc and exert *in vivo* activity: DC-SIGN and CD23 (3, 4). Other C-type lectins with similar binding capacity have been also suggested, including CD22 and DCIR; however, their exact role *in vivo* has not been clearly defined yet (80–82).

DC-SIGN, also referred as CD209, is a C-type lectin that is encoded by the *CD209* gene and is expressed primarily by dendritic cells, macrophages, and monocytes (83, 84). Similar to other C-type lectins, DC-SIGN binds to a number of carbohydrate ligands, predominantly mannose-rich glycan structures that are commonly found on the surface of heavily-glycosylated proteins as well as bacterial and viral glycoproteins, like the HIV-1 envelope glycoprotein, gp160 (85). Given the diversity and the differential binding affinities of the molecules that have the capacity for DC-SIGN engagement, little is known about the exact

signaling events initiated upon ligand binding. Downstream signaling cascades induced following receptor crosslinking appear to be highly dependent upon the type and the nature of the DC-SIGN – ligand interaction, as well as the effector leukocyte types involved. In the case of sialylated IgG Fc – DC-SIGN interaction, receptor engagement on regulatory macrophages induces the expression of interleukin 33 (IL-33) that limits T cell- and IgG-mediated inflammation (86, 87). Secreted IL-33, a potent Th2-polarizing cytokine known for its pleiotropic immunomodulatory effects, promotes Treg activation and expansion that effectively suppresses Th1 and Th17 responses (87). Additionally, IL-33 stimulates the release of IL-4 by basophils that in turn induces the upregulation of the inhibitory type I Fc γ R, Fc γ RIIb on effector inflammatory monocytes and macrophages at sites of inflammation to limit IgG-mediated inflammation, by balancing the activity of activating type I Fc γ Rs (86).

CD23 is also a heavily glycosylated C-type lectin that is encoded by the *FCER2* gene, which is located at the same locus with CD209 (19p13), probably reflecting the similarities in their function and activity between these two receptors. CD23 exists in two splicing isoforms: CD23a and CD23b, with no structural or functional differences. CD23a is constitutively expressed by mature B cells, whereas CD23b is only expressed upon induction by IL-4 by several leukocyte types, including T cells, monocytes, macrophages and granulocytes (88). CD23 has been initially identified as the low affinity receptor for IgE. Indeed, it has the capacity to bind IgE with low affinity (K_D : 10^{-7}); a property attributed to the intrinsic flexibility of the C ϵ 3 domain of IgE (3, 89, 90). Molecular modelling revealed that sialylated IgG Fc also adopts increased flexibility that allows for binding to CD23 in analogy to the IgE:CD23 complex (3). Binding of IgE-containing immune complexes to CD23 on B cells has been shown to regulate IgE production by B cells, as well as to control antigen capture and presentation to dendritic cells (91, 92). CD23 – sialylated IgG interactions has been recently demonstrated to participate in the regulation of IgG affinity maturation and responses by inducing the expression of the inhibitory type I Fc γ R, Fc γ RIIb on B cells (32).

Fc γ R-IgG interactions in passive immunization

Passive immunization was the first effective therapeutic intervention against a number of infectious diseases, based on seminal work by Emil von Behring and Shibasaburo Kitasato during the late 19th century (93, 94). For over five decades, passive administration of anti-serum had been used with remarkable success to prevent or treat bacterial and viral infections. However, the systematic use of antibody therapy has been largely abandoned, mainly due to the lot-to-lot variability of the polyclonal antibody preparations, the relative difficulty of administration over conventional chemotherapeutic agents, and the reported side effects. Remarkable advances in hybridoma technologies over the past decades have revolutionized the approaches for the generation of highly specific monoclonal antibodies with exceptional *in vivo* activity. Currently, there are over 40 FDA-approved monoclonal antibodies in clinical use and more than a hundred in clinical trials. Indeed, antibody-based therapeutics are the first line of treatment for a number of chronic inflammatory and neoplastic disorders and have demonstrated unsurpassed efficacy and safety compared to conventional therapeutic approaches (95). Monoclonal antibody-based therapeutics have been developed primarily for the treatment of neoplastic diseases, including lymphoma and

several types of solid tumors (96, 97). Additionally, a number of monoclonal antibodies exist that have been successfully used for the control of chronic inflammatory diseases, by directly targeting key inflammatory molecules, such as interleukins and TNF- α , as well as for the prophylaxis or therapy of a range of infectious diseases, including RSV, anthrax and Ebola (98–100).

Apart from the observed safety and efficacy of antibody-based therapeutics, their widespread clinical use has provided substantial evidence supporting a role for Fc γ R effector function in their *in vivo* activity. A number of genetic association studies indicated a strong correlation of allelic variants of type I Fc γ Rs with clinical outcome following treatment with anti-tumor monoclonal antibodies. Indeed, patients carrying the allelic variants of Fc γ RIIa and Fc γ RIIIa that exhibit increased affinity for human IgG subclasses demonstrated greater responsiveness to anti-tumor antibody therapy in cases of B cell lymphomas, breast and colorectal cancers (101–106). Similarly, IgG Fc domain engineering for enhanced binding to the activating type I Fc γ R, Fc γ RIIIa resulted in a significant improvement in the therapeutic activity of anti-CD20 monoclonal antibodies (27). These clinical observations strongly suggest a key role for Fc γ R-mediated pathways in the *in vivo* activity of antibodies. A number of *in vivo* studies have dissected the exact mechanisms of Fc effector pathways and suggested novel approaches for optimizing the *in vivo* efficacy of passively administered antibodies in tumor and infectious disease models (12, 19, 21, 107, 108).

As described in the previous section, the outcome of IgG-mediated inflammation and immunity is the result of the balancing activity of activating and inhibitory type I Fc γ Rs expressed by the various effector leukocyte types. This hypothesis has been initially tested in the mouse Fc γ R system in a model of lung metastasis of melanoma cells. Based on the differential capacity of mouse IgG subclasses to engage mouse activating type I Fc γ Rs, IgG subclass variants of an antibody targeted against a glycoprotein (gp75) expressed by melanoma cells were generated and their *in vivo* activity was compared (12). The cytotoxic activity of the anti-gp75 antibodies perfectly correlated with their capacity for engaging activating type I Fc γ Rs. In particular, mouse IgG2a subclass variants demonstrated augmented *in vivo* activity compared to mouse IgG2b, while the mouse IgG1 variant exhibited minimal activity. This hierarchy reflects the capacity of the mouse IgG subclasses to interact with the different classes of mouse Fc γ Rs. Mouse IgG2a preferentially engages the activating Fc γ RIV (orthologue of human Fc γ RIIIa) with 100-fold greater affinity compared to the inhibitory Fc γ RIIb, whereas mouse IgG1 preferentially engages Fc γ RIIb, with minimal affinity for the activating Fc γ RIV (12, 109). These results have clearly demonstrated that the *in vivo* activity of antibodies is highly dependent on their capacity to engage activating Fc γ Rs and suggested that manipulation of the Fc γ R-mediated pathways could greatly influence the *in vivo* protective activity.

Similar findings suggesting a role for Fc γ Rs in the *in vivo* activity of monoclonal antibodies were observed for a number of different models ranging from tumors to bacterial (*Bacillus anthracis* (20, 107), *Streptococcus pneumoniae* (110), *Staphylococcus aureus* (111)), fungal (*Cryptococcus neoformans* (112, 113)) and viral (HIV-1 (19, 114, 115), influenza (108, 116), RSV (28)) infections. Common to all these models was the absolute requirement for Fc effector function for optimal antibody *in vivo* activity. Although the precise effector

functions initiated upon activating type I Fc γ R engagement by IgG Fc differ between the various experimental models, it is anticipated that Fc γ R-mediated cellular activation of effector leukocytes, such as monocytes, macrophages, neutrophils, and NK cells, would result in antibody-dependent cellular cytotoxicity, phagocytosis, cytokine and chemokine release. Such effector functions would facilitate the cytotoxic lysis and clearance of tumor or infected cells, promote microbial opsonization and clearance, as well as enhance host immune responses, by altering the survival, mobilization and differentiation of leukocyte populations. Using examples from an infectious (HIV-1) and a neoplastic (CD20⁺ B cell lymphoma) disease, in the following sections we will discuss in detail the role of Fc γ R-mediated interactions in the *in vivo* protective activity of therapeutic antibodies.

HIV-1 passive immunization

The development of broadly neutralizing antibodies against HIV-1 and their clinical use for the prevention or treatment of the infection has been hampered by the unique immune evasion mechanisms of HIV-1. Indeed, HIV-1 displays remarkable diversity (117, 118), its main envelope glycoprotein is shielded in an elaborate glycan structure that masks all sites of vulnerability and it generally presents limited immunogenicity (119, 120). These structural and functional characteristics of the HIV-1 virus limit the host's capacity to mount sustained antibody responses with broad neutralizing activity. However, a small fraction of patients develop potent neutralizing antibodies with broad activity against other viral clades that can efficiently suppress viremia for several years (121, 122). By B cell cloning of neutralizing antibodies from these patients, several broadly neutralizing anti-HIV-1 antibodies targeting virus' envelope glycoprotein have been isolated and extensively characterized over the past 5 years (122). Passive administration of these antibodies confers protection against SHIV challenge in macaques (114, 123) and HIV-1 infection in humanized mice (124). Additionally, these broadly neutralizing antibodies have been shown to provide durable and sustained suppression of viremia in chronically SHIV-infected macaques (125, 126), and in humanized mice with established HIV-1 infection (19, 115, 127, 128). These encouraging findings suggest the potential clinical use of broadly neutralizing anti-HIV-1 antibodies for the prevention and treatment of HIV-1 infection in humans. Indeed, in a recent phase I/II trial of the broadly neutralizing anti-CD4bs antibody, 3BNC117, effective suppression of viremia in chronically infected HIV-1 patients was observed that lasted for several days post-antibody administration (129).

A few previous studies have suggested a role for Fc γ Rs during HIV-1 infection. In a small patient cohort, the clinical progression of AIDS was found to be associated with genetic variants of Fc γ RIIa that affect IgG binding affinity (130). Similarly, the *in vivo* activity of the neutralizing antibody b12 has been shown to depend on Fc γ R, but not on complement interactions in a model of pre-exposure prophylaxis in SHIV-challenged Rhesus macaques (114). Given the recent isolation of anti-HIV-1 antibodies with potent and broad neutralizing activity, it was not until recently that the mechanisms of Fc effector function that contribute to their *in vivo* activity were investigated. Using models of HIV-1 entry (131), IgG subclass variants with preferential binding capacity for activating type I Fc γ Rs exhibited improved *in vivo* activity and induced enhanced clearance of circulating virus particles (19). Similarly, in studies using HIV-1-infected humanized mice, the *in vivo* activity of broadly neutralizing

antibodies has been shown to depend on Fc-Fc γ R interactions (19, 115). In particular, IgG Fc domain variants with diminished capacity to engage all classes of type I Fc γ Rs failed to adequately suppress viremia in models of antibody-mediated post-exposure prophylaxis or therapy (19, 115). Likewise, Fc domain variants of anti-HIV-1 antibodies engineered for enhanced binding to activating type I Fc γ Rs exhibited improved *in vivo* activity, as evidenced by prolonged and durable suppression of viremia in humanized mice with established HIV-1 infection (19). Collectively, these results highlight a key role for Fc effector function in the *in vivo* activity of anti-HIV-1 broadly neutralizing antibodies and suggest approaches for the development of improved antibody-based therapeutics with augmented *in vivo* efficacy through enhanced Fc-Fc γ R interactions.

Passive Immunotherapy of B-cell lymphoma

Several monoclonal antibodies are currently approved and used for the treatment of neoplastic diseases. The anti-tumor activity of these antibodies is achieved by directly targeting tumor cells through cytotoxic lysis, inducing cell death or blocking cell survival, antagonizing the activity of growth factors or their receptors, as well as altering the tumor microenvironment to stimulate host anti-tumor responses (96, 97). Anti-CD20 antibodies, like rituximab were among the first to be approved for cancer patients and have been used for several years to treat various classes of CD20⁺ leukemias and lymphomas as well as to control autoimmune syndromes.

A number of different mechanisms that account for the activity of anti-CD20 antibodies have been suggested, including induction of B cell apoptosis and complement-mediated cytotoxicity; however, *in vivo* evidence from animal and human studies strongly supports a dominant role for Fc γ R-mediated effector activity (103, 105, 132, 133). For example, allelic variants of the activating type I Fc γ Rs, Fc γ RIIa and Fc γ RIIIa that exhibit altered affinity for human IgG have been shown to be associated with the efficacy of CD20⁺ cell depletion in lymphoma patients (103, 105). The requirement for activating type I Fc γ Rs to mediate the *in vivo* activity of anti-CD20 antibodies has also been demonstrated in mouse models of B-cell lymphoma. Deletion of the FcR γ -chain that is required for the expression and signaling function of activating type I Fc γ R in mice, abrogated the *in vivo* activity of rituximab (134). Similarly, *Fcgr2b*-knockout mice that lack the inhibitory Fc γ RIIb exhibited improved therapeutic responses following anti-CD20 antibody administration (134). These findings have been recently extended in studies using mice humanized for type I Fc γ Rs (21). These mice faithfully recapitulate the unique pattern of human Fc γ R expression and have been successfully used for the pre-clinical evaluation of cytotoxic antibodies in several models (18, 20, 108). A panel of Fc domain-engineered anti-CD20 antibodies were generated that exhibited selective binding to particular classes of human Fc γ Rs and their activity was assessed in Fc γ R-humanized mice challenged with human CD20-expressing lymphoma cells (21). Fc domain variants with selectively enhanced binding either to all the classes of activating type I Fc γ Rs or to the activating Fc γ RIIIa exhibited improved anti-tumor activity, achieving tumor cell clearance over a short time period. Investigation into the Fc γ RIIIa-mediated mechanisms that contributed to the antibody-dependent tumor clearance revealed that Fc γ RIIIa-expressing macrophages were the dominant effector cell population that mediated the anti-tumor effect, possibly through phagocytic processes (21).

Similarly, the importance of activating type I Fc γ R_s in the *in vivo* activity of anti-CD20 antibodies is further highlighted by the recently approved anti-CD20 monoclonal antibody, obinutuzumab (27). This antibody exhibits the same antigen specificity as rituximab; however, it displays improved binding to the activating Fc γ RIIIa through engineering of the Fc domain-associated N-linked glycan (23, 26). Comparison of the therapeutic activity of this Fc-optimized antibody with the parental, unmodified one (rituximab) in chronic lymphocytic leukemia patients revealed that obinutuzumab extended progression-free survival for over 10 months compared to rituximab (27). These findings provide a great example on how the *in vivo* efficacy of a therapeutic antibody can be effectively improved through the study of the underlying mechanisms that govern Fc-Fc γ R interactions.

Fc γ R-mediated pathways in active immunization

As discussed in the previous sections, engagement of type I and type II Fc γ R_s by IgG immune complexes can have diverse downstream consequences that initiate a number of immunomodulatory effector functions. Such functions not only provide immediate cytotoxicity and clearance of IgG-coated targets, but have also the capacity to modulate the immune responses through a range of effects on the various leukocyte types, including the differentiation and activation of antigen-presenting cells (APCs), cellular activation and release of chemokines and cytokines by innate effector leukocytes, enhanced antigen uptake and presentation by APCs, modulation of T cell activation by APCs, and regulation of their entry into the germinal centers, modulation of affinity maturation by Fc γ R-bound immune complexes retained on follicular dendritic cells, and finally plasma cell survival and regulation of antibody production (4).

These functions highlight the potential of Fc γ R-mediated pathways to modulate the adaptive immune response and have been exploited in immunization strategies, which employ immune complexes consisting of antigen-specific antibodies in combination with antigen to enhance the host immune responses against infectious agents and tumor antigens. Indeed, the concept of IgG-mediated enhancement of T-cell and antibody responses can be traced back to as early as the end of the 19th century in immunization studies against diphtheria (135). Additionally, substantial evidence over the course of years clearly suggests that immunization with IgG immune complexes greatly enhances the immunogenicity of an antigen and improves host immune responses against a number of microbes, including Venezuela equine encephalitis virus, Newcastle disease virus, infectious bursal disease virus and HIV-1 (136–142). Indirect evidence supporting a role for Fc-Fc γ R interactions in modulating adaptive immune responses has come from passive administration of therapeutic antibodies to humans and non-human primates for the treatment of neoplastic and infectious diseases. For example, the treatment of non-Hodgkin lymphoma patients with anti-CD20 antibodies often results in the generation of a ‘vaccinal’ response such that relapsed patients who are re-treated with the same anti-tumor antibody show rapid and enhanced responses when compared to their response to initial therapy (143, 144). The basis for this vaccinal effect has been attributed to the development of robust anti-tumor CD8 responses in these patients, induced by the generation of immune complexes between the passively administered anti-CD20 antibody and the tumor cells (143). In another example, two independent studies using non-human primates demonstrated the contribution of passively

administered anti-HIV-1 neutralizing antibody to the subsequent development of a host antiviral response with protective and sustained activity (145, 146). Administration of the neutralizing anti-HIV-1 antibody b12 in infected animals resulted in the modulation of their B cell responses and accelerated the production of neutralizing antibodies. Hyperimmune sera from these animals exhibited potent activity against SHIV and SIV infection when passively administered into naïve animals (145, 146). Similar augmentation in B cell responses was also observed following administration of anti-HIV-1 broadly neutralizing antibodies in chronically SHIV-infected rhesus monkeys (125).

Although these studies strongly suggest that active immunization with IgG immune complexes results in enhanced antigen immunogenicity and augmented humoral and cellular immune responses, the precise mechanisms of this phenomenon are poorly characterized. Several potential regulatory pathways have been suggested that could participate in the Fc γ R-mediated enhancement of adaptive immunity. Dendritic cells, follicular dendritic cells and macrophages are the dominant immune cell populations that could influence antibody responses through antigen presentation and activation of lymphoid cells. Activating type I Fc γ Rs actively participate in the uptake of IgG immune complexes through endocytic and phagocytic processes (59, 147). Internalized immune complexes are efficiently processed and presented on MHC class I and class II molecules by dendritic cells, leading consequently to enhanced CD4⁺ and CD8⁺ T cell-mediated responses (4, 148)(Figure 3). Additionally, engagement of activating type I Fc γ Rs expressed on the surface of dendritic cells induces cell maturation, upregulation of MHC and co-stimulatory molecules, and consequently enhanced antigen presentation (148–150). Likewise, the function of the activating type I Fc γ Rs is balanced by the inhibitory Fc γ RIIb, as genetic deletion or blockade of Fc γ RIIb leads to spontaneous DC maturation and influences macrophage polarization (149, 151, 152). Studies using dendritic cells from *Fcgr2b*^{-/-} mice, revealed a critical role for Fc γ RIIb in controlling IgG-mediated maturation of dendritic cells (149). Whereas IgG immune complexes are efficiently captured by dendritic cells, they fail to induce DC maturation without additional stimulatory signals (e.g. TLR signaling), as co-engagement of Fc γ RIIb sufficiently blocks signaling from the activating type I Fc γ Rs (149). The potential of activating Fc γ R engagement on dendritic cells to induce T cell responses has been recently explored using Fc γ R-humanized mice in a model of human CD20⁺ lymphoma (21). Under homeostatic conditions, human dendritic cells (e.g. monocyte-derived dendritic cells) express only one class of activating type I Fc γ R, Fc γ RIIa, and its activity is controlled by the inhibitory Fc γ RIIb (18). In this study, investigation into the mechanisms underlying the induction of T cell responses following treatment with anti-CD20 antibodies revealed an absolute requirement for Fc γ RIIa expression by dendritic cells in the generation of T cell responses against CD20 (21). Indeed, Fc engineered variants of anti-CD20 antibody with enhanced affinity for Fc γ RIIa exhibited improved IgG-mediated T cell responses, by increasing the threshold for Fc γ RIIb inhibition (21). These studies strongly suggest that balancing activating and inhibitory type I Fc γ Rs on antigen presenting cells, like dendritic cells is a key regulatory process that greatly influences subsequent immune responses (Figure 3).

Fc γ R engagement on B cells by IgG immune complexes also induces a number of immunomodulatory processes with the potential to influence B cell selection and

consequently the antibody affinity and production. B cells express only the inhibitory Fc γ RIIb throughout development, and not any other type I Fc γ Rs. Despite the lack of activating type I Fc γ Rs to counterbalance the activity of Fc γ RIIb, Fc γ RIIb engagement induces differential functional responses on B cells and several studies have highlighted its key regulatory role in shaping the antibody response and maintaining tolerance (78). Exclusive engagement of B cell Fc γ RIIb induces pro-apoptotic signals, whereas co-ligation of Fc γ RIIb with the B cell receptor attenuates these signals, leading to B cell survival (73, 77). This mechanism drives B cell selection, as B cells with higher affinity for the antigen are selected, whereas those with receptors exhibiting low or no affinity undergo apoptosis. Indeed, Fc γ RIIb-deficient mice fail to generate high affinity antibody responses, likely due to defects in B cell selection mechanisms (78). Loss of the B cell receptor expression in plasma cells leads to unopposed, pro-apoptotic signaling through Fc γ RIIb engagement, which represents a homeostatic regulatory mechanism to control IgG production by plasma cells (153). Engagement of Fc γ RIIb (human or mouse) on plasma cells by IgG immune complexes during an ongoing immune response acts as a negative feedback mechanism to prevent uncontrolled IgG production.

Apart from Fc γ RIIb, B cells also express the type II Fc γ R, CD23 at variable levels during B cell maturation, which is also part of a novel regulatory pathway that modulates B cell activation and affinity maturation (32)(Figure 4). Studies using IgE immune complexes have shown that CD23 engagement is required for the IgE-mediated enhancement of antibody and T cell responses (91). Likewise, CD23 contributes to the capture of IgE immune complexes by B cells and to their transfer to CD11c⁺ antigen presenting cells, leading to enhanced CD4⁺ T cell immunity (92). Given the capacity of CD23 to also interact with sialylated IgG Fc (3), a similar mechanism likely modulates immune responses through CD23 engagement by sialylated IgG immune complexes. Indeed, in a recent study, a key role for sialylated IgG Fc-CD23 interactions has been presented with great implications for the development of novel immunization strategies (32). Interaction of sialylated IgG immune complexes with CD23 upregulates Fc γ RIIb expression on B cells, which in turn leads to increased threshold of B cell selection. Using a model of influenza hemagglutinin (HA) immunization, vaccination with sialylated Fc anti-HA immune complexes induced higher affinity anti-HA IgG responses, with broadly neutralizing, *in vivo* protective activity (32)(Figure 4). By contrast, immunization of CD23^{-/-} mice or with asialylated anti-HA immune complexes failed to generate high affinity anti-HA antibody responses, supporting thereby a key role for the sialylated IgG Fc-CD23-Fc γ RIIb pathway in modulating B cell selection and antibody affinity maturation (32).

Concluding Remarks

The immunomodulatory effects of the IgG Fc domain interactions with type I and type II Fc γ Rs have the potential to dramatically influence the outcome of IgG-mediated inflammation and immunity. In a process primarily determined by the intrinsic flexibility of the IgG Fc domain that is tightly regulated by the Fc-associated glycan structure and composition, engagement of the different types of Fc γ Rs modulates the activity of several distinct regulatory pathways that mediate Fc effector functions.

The development and clinical use of passive immunization strategies against several infectious and neoplastic diseases support a dominant role for Fc γ R-mediated pathways in the *in vivo* protective activity of antibody-based therapeutics. Manipulation of Fc-Fc γ R interactions through Fc domain engineering has already led to the development of a new generation of monoclonal antibodies with improved *in vivo* efficacy through augmented Fc effector activity. Likewise, a number of studies strongly suggest a significant role for Fc γ R engagement by IgG immune complexes in the modulation of adaptive immune responses. Dissection of the precise mechanisms that drive IgG-mediated enhancement of cellular and humoral immunity would lead to the development of novel active immunization approaches that rely on the capacity of type I and type II Fc γ Rs to regulate immune responses.

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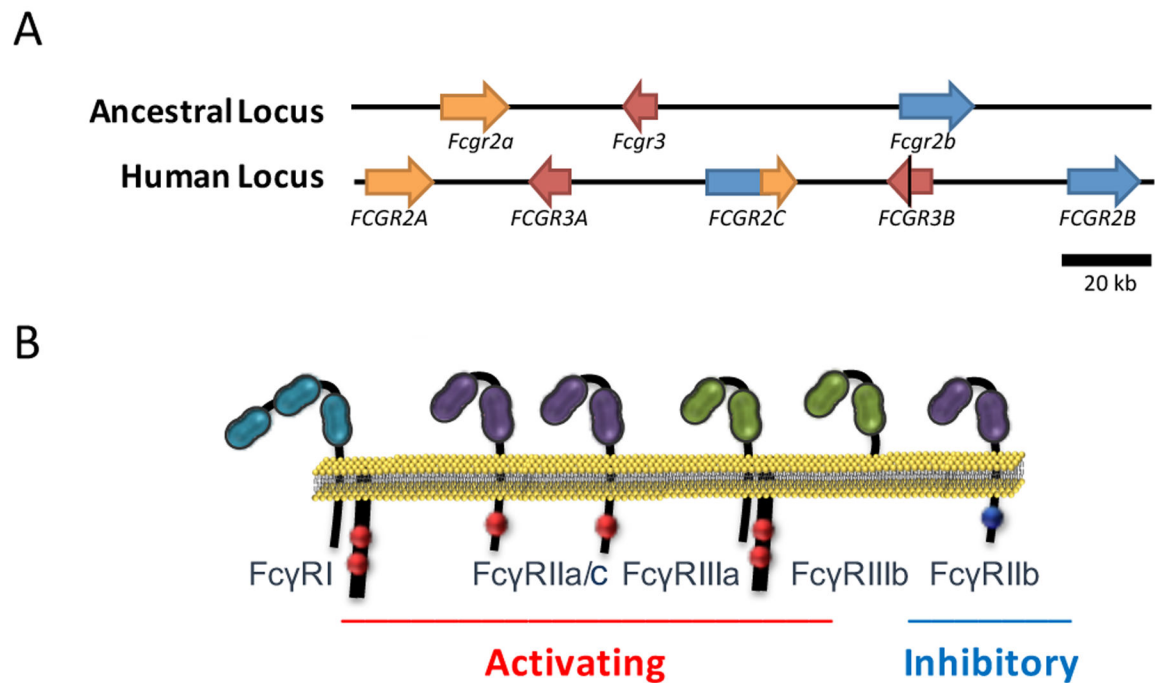


Figure 1: Structure and characteristics of the human type I Fc γ R family.

(A) The main human Fc γ R locus is located at *1q23* and comprises all the genes encoding the low affinity type I Fc γ Rs. The unique genomic organization of the human Fc γ R locus is the result of a non-homologous recombination event of the ancestral Fc γ R locus, which is highly conserved among several mammalian species. This event gave rise to additional Fc γ R-encoding genes that are uniquely found in humans. (B) Human type I Fc γ Rs are functionally categorized into activating or inhibitory based on the presence of an intracellular ITAM or ITIM motif. Engagement of activating or inhibitory Fc γ Rs by IgG immune complexes induces immunostimulatory or immunosuppressive signals, respectively, influencing thereby the outcome of IgG-mediated inflammation and immunity.

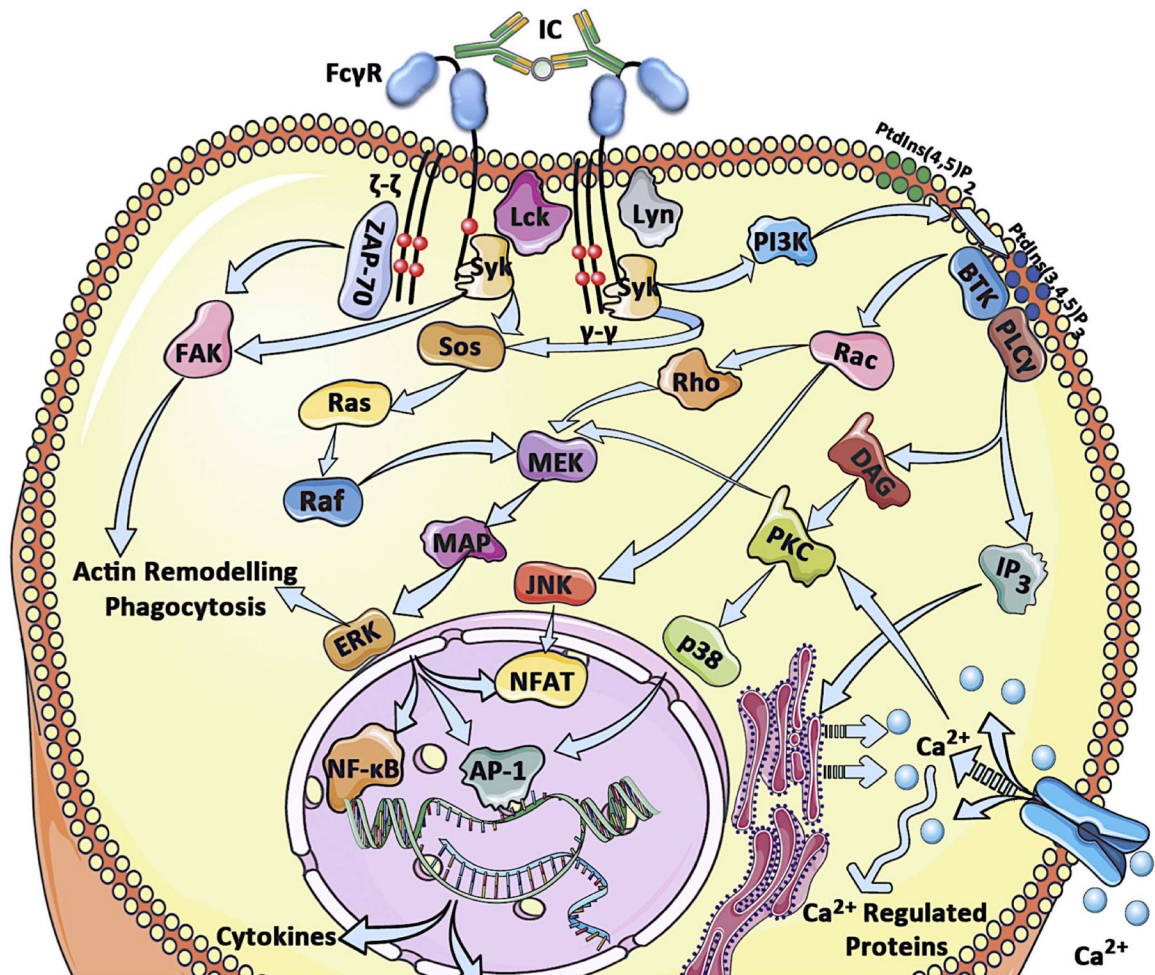


Figure 2: Overview of the downstream signaling events following crosslinking of activating type I FcγRs.

Engagement of activating type I FcγRs by IgG immune complexes triggers receptor crosslinking and phosphorylation of the ITAM domains by Src family kinases. This event subsequently initiates a cascade of signaling pathways that result in cellular activation, Ca²⁺ mobilization, activation of kinases involved in actin remodeling and at later stages transcription factor activation and expression of cytokines, chemokines and survival proteins that further augment inflammatory processes.

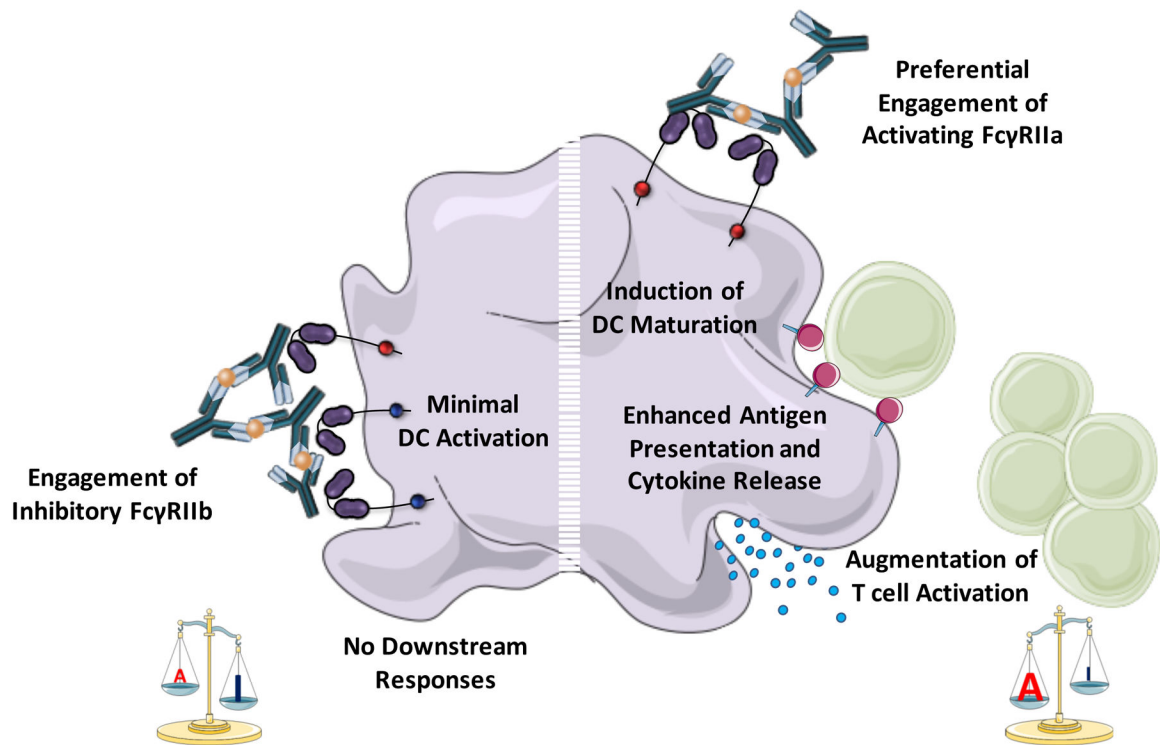


Figure 3: The role of FcγR-mediated pathways in dendritic cell function.

Human dendritic cells (DC) express two classes of type I FcγRs: the activating FcγRIIa, and the inhibitory FcγRIIb. Balancing the activity of these receptors is critical for DC function and for the regulation of T cell activation. FcγR engagement by IgG immune complexes fail to stimulate DC activation, as any pro-inflammatory signals initiated by FcγRIIa are counterbalanced by FcγRIIb. However, preferential engagement of the activating FcγRIIa (either through Fc domain engineering or FcγRIIb deletion or blockade) induces DC maturation and the upregulation of MHC and co-stimulatory molecules, leading to enhanced antigen presentation, and augmented T cell activation.

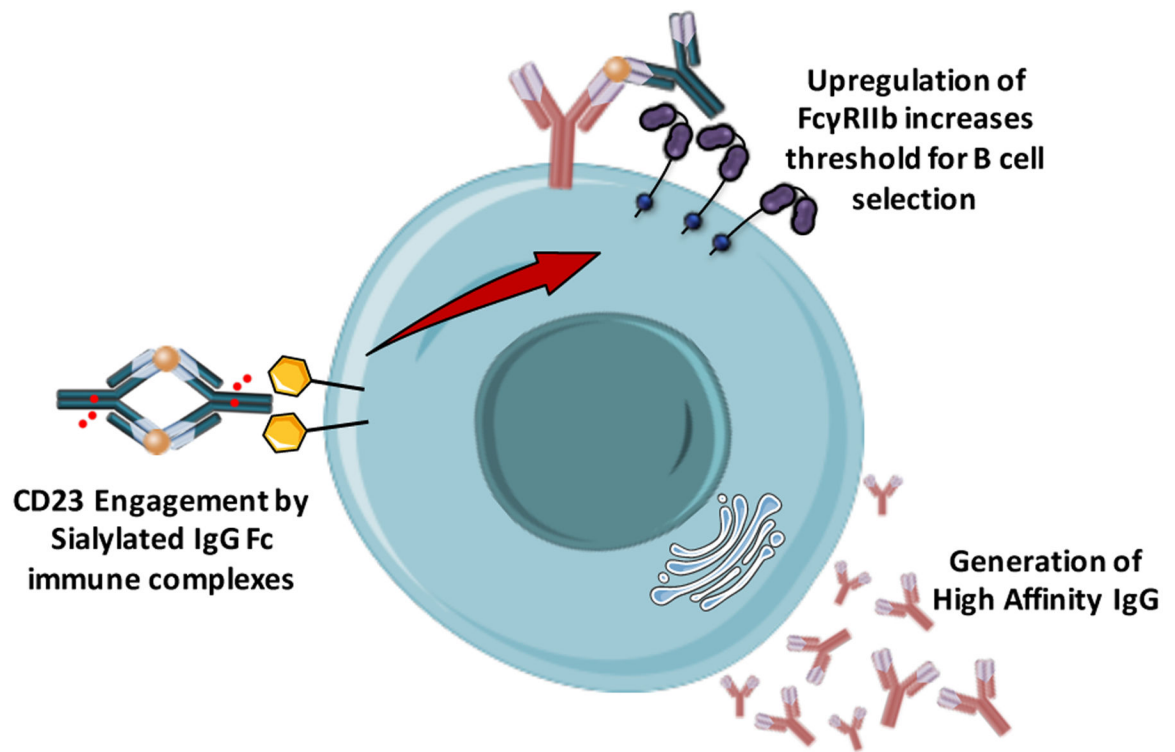


Figure 4: Regulation of antibody responses through a CD23-FcγRIIb pathway.

Sialylated IgG immune complexes interact with CD23 expressed by B cells, leading to the upregulation of FcγRIIb. In turn, increased FcγRIIb expression raises the threshold for B cell selection and B cells with higher affinity B cell receptor are selected over those with lower or no affinity. This process eventually leads to the generation of higher affinity antibody responses with potent *in vivo* activity.