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Jeffrey V. Ravetch
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# Fc Mediated Activity of Antibodies

## Structural and Functional Diversity

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Responsible Series Editor: Rino Rappuoli

Springer

*Editors*
Jeffrey V. Ravetch
Department of Leonard Wagner Laboratory
of Molecular Genetics and Immunology
Rockefeller University
New York, NY, USA

Falk Nimmerjahn
Department of Biology
University of Erlangen-Nürnberg
Erlangen, Bayern, Germany

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# Preface

If one looks through the lists of novel drugs targeting cancers, autoimmune/chronic inflammatory diseases, and infection, a molecule appearing very (if not most) frequently on that list will be antibodies of the Immunoglobulin G (IgG) isotype. Indeed, the much celebrated breakthrough in cancer immunotherapy is based on intact antibodies or antibody fragments, such as bispecific T cell engaging antibodies (BiTEs); and chimeric antigen receptors (CAR), which are antibody fragments expressed on T cells. Thus, antibodies are the new and old kid on the block for immunotherapy and surely will be in the focus of novel therapeutic approaches for many years to come. An in depth understanding of how antibodies mediate their activity is therefore of critical importance. Research of many groups over the last three decades has provided clear evidence that IgG binding to cellular Fc-receptors is essential for the activity of antibodies in vivo, emphasizing that the antibody constant region is much more than a framework carrying the variable domain, but rather is the essential—and actually not so constant—linker responsible for triggering IgG effector functions.

In autoimmunity, IgG antibodies play several important roles. Firstly, as autoantibodies they are drivers of autoimmune inflammation. Again, Fc-receptors have been identified as central players responsible for triggering the pro-inflammatory effects of autoantibodies. Second, IgG antibodies are used in the form of monoclonal antibodies targeting key pro-inflammatory cytokines or self-reactive immune cells. Finally, the infusion of polyclonal serum IgG preparations pooled from thousands of donors, also called intravenous IgG therapy (IVIg), is efficiently able to suppress a wide variety of chronic and acute autoimmune diseases, prompting many groups to investigate its mechanism of action and solve the mystery of IVIg activity. While many potential mechanisms of action of IVIg have been proposed over the years, one completely unexpected finding was that the sugar moiety attached to the IgG Fc-domain seemed to play a crucial role for the anti-inflammatory activity of these polyclonal IgG preparations. More in depth studies provided convincing evidence that especially IgG glycoforms carrying terminal sialic acid residues were responsible for the anti-inflammatory activity and that a family of type II Fc-receptors was involved in this

anti-inflammatory pathway. A side effect of these observations was that it prompted many groups to re-visit the impact of glycosylation on immunoglobulin activity more broadly. This has led to the unexpected finding that not only IgG but also IgE antibody activity is regulated through glycosylation. Of note, especially one of the many sugar moieties attached to IgE was shown to be of critical importance for productive binding of IgE to its Fc-receptor. Coming to IgG and infection, IgG antibodies are essential to protect the host from infection or from the detrimental activity of bacterial toxins. Again, Fc-receptors were shown to be of major importance also for the activity of neutralizing antibodies, which were previously thought to mediate their activity independently of Fc-receptors. But antibodies are clearly not only beneficial in infection. Very recent studies have emphasized that glycovariants of virus specific IgG antibodies lacking fucose residues may be responsible for enhancing Dengue virus infection and pathology in hosts, also known as antibody dependent enhancement of infection. As IgG glycovariants lacking fucose modulate IgG binding to human FcgRIIIa (in humans) and FcgRIV (in mice), this again pinpoints towards an important role of Fc-receptors in this process. Finally, in a world of fading activity of antibiotics against bacteria more successful vaccination strategies will be of major importance. Harnessing the host through generating a most productive antibody response against microorganisms will be key to cope with this global threat. Using antibodies in combination with the antigens (that is: immune complexes) to boost immune responses is a very efficient and in fact the most natural way of enhancing immunity. Again Fc-receptors present on dendritic cells play a key role for such novel vaccination strategies.

In this book, we were able to bring together an exciting list of experts in the field covering all of these hot topics of antibody and Fc-receptor research. Stylianos Bournazos will look at Fc-receptors from an evolutionary point of view, while Graziano and Engelhardt will discuss the role of Fc-receptors for immunotherapy of cancer. The chapters of Beneduce and colleagues, Wang, and Shade and colleagues will discuss the importance of IgG glycosylation for the pro- and anti-inflammatory activities of IgG and IgE. Finally, the two chapters by Wen and Shi and Wieland and Ahmed will focus on how Fc-receptors impact vaccination and infection.

Naturally the examples mentioned above are only a fraction of the research, which is currently ongoing in the field of antibodies and Fc-receptors, but they clearly highlight how broad the impact of research on Fc-mediated activities of antibodies can be. We would like to thank all authors contributing to this volume again for their excellent contributions and we hope that it will stimulate new research ideas in this exciting area of immunology.

New York, USA — Jeffrey V. Ravetch
Erlangen, Germany — Falk Nimmerjahn

# Contents

# IgG Fc Receptors: Evolutionary Considerations


**Stylianos Bournazos**

## Contents



**Abstract** Immunoglobulins (Ig), a critical component of the adaptive immune system, are present in all jawed vertebrates and through sophisticated diversification mechanisms are able to recognize antigens of almost infinite diversity. During mammalian evolution, IgG has emerged as the predominant Ig isotype that is elicited upon antigenic challenge, representing the most abundant isotype present in circulation. Along with the IgG molecule, a family of specialized receptors has evolved in mammalian species that specifically recognize the Fc domain of IgG. These receptors, termed Fcγ receptors (FcγRs), are expressed on the surface of effector leukocytes and upon crosslinking by the IgG Fc domain mediate diverse immunomodulatory processes with profound impact on several aspects of innate and adaptive immunity. FcγRs share a high degree of sequence homology among mammalian species and the ancestral locus, where the genes that encode for FcγRs are mapped, can be traced back early in mammalian evolution. FcγRs also share a number of common structural and functional properties among mammalian species and utilize highly conserved motifs for transducing signals upon engagement. Despite the high homology of FcγRs in diverse mammalian species, human FcγRs exhibit unique features relating to the gene organization, expression pattern in the

S. Bournazos (✉)
The Laboratory of Molecular Genetics and Immunology, The Rockefeller University, 1230 York Ave, New York, NY 10065, USA
e-mail: sbournazos@rockefeller.edu

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various leukocyte populations, as well as affinity for human IgGs. Such inter-species differences in FcγRs biology between humans and other mammalian species represents a major limitation for the interpretation of in vivo studies on human IgG function using conventional animal models.

## 1 The Emergence of IgG Antibodies

Complex defense mechanisms are ubiquitously found in all living organisms and are critical for the survival of these organisms in an antigenic world. Immunity is generally mediated by the early reactions of the innate immune system, followed by the later responses of adaptive immunity, characterized by almost infinite diversity and high specificity. Although immune mechanisms featuring the cardinal signs of adaptive immunity, i.e. diversity and specificity, have been discovered in invertebrates and jawless vertebrates, adaptive immunity based on highly diversified antigen-specific receptors is present only in jawed vertebrates. Indeed, key immune components of adaptive immunity, including antigen receptors, such as immunoglobulins (Ig) and T-cell receptors (TCR), as well as antigen-presenting MHC molecules are locked in a coevolving unit that orchestrates clonal selection and MHC-regulated processes during an adaptive immune response.

In addition to their common function, adaptive immune antigen receptors, such as Ig and TCR, share structural similarities, as they both comprise domains of the immunoglobulin superfamily (IgSF). IgSF domains are the building blocks for a large number of molecules across several species of the animal kingdom and represent the most prevalent domain in immune defense molecules. Apart from antigen receptors, IgSF domains are found in numerous receptors and molecules with a key role in cell-cell interactions, cell adhesion, as well as immune cell signaling. Despite the diverse functions of IgSF-containing molecules, these functions are somehow related, as IgSF domains are almost exclusively involved in protein-ligand interactions. The main structural feature of the IgSF domain is the stable shape of a β barrel that comprises two interfacing β sheets that are typically linked by a disulfide bond. Based on the domain constitution of their β strands and loops, IgSF domains are classified into four major types: the variable (V), the two constant domains (C1 and C2), as well as an intermediate type, which shares common features with both the C1 and C2 domains. Among the IgSF domains, the V domain type is the most structurally flexible and complex, featuring more strands, which correspond to the CDR2 region in Ig and TCRs. These unique structural features are critical for mediating highly specific antigenic recognition, accommodating the high degree of sequence diversity that characterizes antigen receptors, such as Ig and TCRs. The final structure of these antigen receptors is achieved through a linear combination of V and C-type IgSF domains. For example, a typical Ig molecule is a heterodimeric structure that comprises two polypeptides of the heavy chain and two of the light chain that are linked via several disulfide bonds forming a macromolecular

complex. Each light chain comprises a single V and a C1 IgSF domain, whereas the Ig heavy chain is formed through the joining of the V-type domain with three-four C1 IgSF domains; the exact number of C domains is variable among the different Ig isotypes.

These unique structural features are highly conserved among all the different Ig isotypes found in diverse species of jawed vertebrates. Indeed, IgM, which is present in all jawed vertebrates, and is thought to represent the primordial Ig isotype, consists of a heavy chain peptide with one V and four C1 domains, which pairs with the light chain (one V and one C1 IgSF domain) through multiple inter-chain disulfide bridges. Monomeric IgM subunits form pentamers or hexamers in the majority of vertebrate species, apart from teleost fish, which form tetramers. Comparative analysis of the Ig isotypes in the jawed vertebrates suggests that a common precursor isotype that is related to IgM and can be traced at the inception of adaptive immunity, gave rise to the various Ig isotypes in jawed vertebrates at specific divergence points during evolution. These Ig isotypes have evolved under constant selection pressure from the challenging antigenic conditions encountered by the various vertebrate species. Indeed, the different Ig isotypes exhibit unique structural and functional characteristics, as well as distinct expression patterns among the various tissue compartments (intestinal, mucosal, serum etc.). For mammalian species, IgG and IgE represent two Ig isotypes that are exclusively found in mammals and mediate effector functions during an adaptive immune response. These isotypes are thought to have emerged from a common, structurally-related ancestor molecule, IgY, which is present in other vertebrate classes, including birds, reptiles, and amphibians, and represents the most abundant, secreted Ig isotype in these species.

## 2 Evolution of FcγR Genes in Mammalian Species

Since the IgG isotype is exclusively found in mammals, receptors for IgG antibodies only exist in mammalian species. These receptors, termed Fcγ receptors (FcγRs), interact with the constant region of the IgG heavy chain heterodimer and have co-evolved in mammalian species along with the emergence of the IgG isotype. For mammals, IgG represents the major Ig isotype in circulation and is abundantly expressed during an adaptive immune response. By directly interacting with IgG antibodies, FcγRs are critical for mediating downstream effector activities with tremendous impact on diverse innate and adaptive pathways. Indeed, despite being a major component of adaptive immunity, IgG antibodies link the innate and adaptive branches of immunity through specific interactions with the FcγRs expressed on the surface of effector leukocytes. Such interactions initiate diverse signaling processes with pleiotropic immunomodulatory functions, which have significant consequences for several aspects of innate and adaptive immunity. Such functions include the regulation of innate leukocyte activation, the uptake of foreign

antigens via phagocytic mechanisms and their processing and presentation to MHC molecules, the regulation of antigen-presenting cell function, the stimulation of cytokine and chemokine synthesis, as well as the selection of high-affinity B-cells, and the regulation of plasma cell survival and consequently IgG production (Bournazos et al. 2017).

Given the diverse immunomodulatory consequences of FcγR engagement by the Fc domain of IgG antibodies, a number of FcγRs have emerged during mammalian evolution, each with distinct signaling activity and function, as well as unique expression profile among the various leukocyte cell types. On the basis of their intracellular signaling motifs and their capacity to induce stimulatory or inhibitory signaling cascades, FcγRs are broadly classified into activating or inhibitory (Nimmerjahn and Ravetch 2005). The major determinant that controls the affinity of an IgG molecule for the various FcγR types is the intrinsic flexibility of the Fc domain of IgG (Pincetic et al. 2014). Similar to the structural characteristics of all Ig isotypes, IgG molecules comprise two heavy and two light chains that are linked into a heterodimeric macromolecular complex through inter-chain disulfide bonds. Each light chain comprises one V-type IgSF domain and a C1-type domain, whereas each heavy chain consists of one V-type domain followed by three C1-type domains, corresponding to the CH1–CH3 domains of the IgG molecule. The characteristic Y structure of the IgG molecule arises from the presence of a hinge domain that separates the Fab domain (CH1 and light chains) from the Fc domain, which comprises the CH2 and CH3 domains. The FcγR binding sites are mapped at the Fc domain at two distinct positions; Type I FcγRs engage the Fc domain at the hinge-proximal region of the CH2 domain, whereas type II FcγRs at the CH2–3 interface (Pincetic et al. 2014). FcγR binding is accomplished through the characteristic horseshoe-like conformation of the Fc domain, which is formed by the tight association of the two CH3 domains at the C-terminal proximal region of the IgG molecule, while the two CH2 domains remain spatially separated. This characteristic tertiary structure of the Fc domain is achieved through the presence of an N-linked glycan conjugated at the amino acid backbone of the CH2 domain of the two heavy chains. The amino acid residue, where this glycan is conjugated is highly conserved in all IgG subclasses from all mammalian species, highlighting the importance of this glycan structure in the regulation of the Fc domain structure and consequently in Fc-FcγR interactions. This Fc-associated glycan resides within the hydrophobic cleft formed by the two CH2 domains and its composition regulates the conformational flexibility of the Fc domain and its capacity to interact with the various type I and type II FcγRs (Sondermann et al. 2013). Indeed, the presence of this glycan structure is critical for maintaining the Fc domain structure permissive for Fc-FcγR interactions, as loss of this glycan either through enzymatic removal or mutation of the amino acid residue where this structure is conjugated diminishes the affinity of the IgG Fc domain for all FcγR types (Albert et al. 2008; Lux et al. 2013).

Although the presence of the Fc-associated glycan structure is critical for maintaining the conformational structure of the Fc domain, the precise composition

of the Fc glycan represents a key regulatory mechanism that controls the affinity of the Fc domain for the various FcγR classes. More specifically, the Fc-associated glycan structure consists of a core biantennary heptasaccharide moiety, which can be modified through the conjugation of additional saccharide units (fucose, galactose, N-acetylglucosamine, sialic acid), thereby yielding numerous variants with differential capacity to modulate Fc domain flexibility and consequently the affinity for the various FcγR types (Bournazos et al. 2017). Analysis of the Fc-associated glycan structure in circulating IgG antibodies has previously revealed substantial heterogeneity with specific Fc glycoforms becoming enriched upon infection and vaccination, in metabolic disease, during pregnancy, as well as during the remission/relapse phases of autoimmune disorders (Anthony et al. 2012; Nakagawa et al. 2007; Scherer et al. 2010; Theodoratou et al. 2016; Wang et al. 2015). Apart from the structure and composition of the Fc-associated glycan, differences in the primary amino acid sequence between the various IgG subclasses represent an additional level of regulation for Fc-FcγR interactions. Although protein homology between subclasses is typically over 95%, differences that exist at the hinge proximal region of the CH2 domain, where the interface for Fc-FcγR interactions is mapped, account for the differential binding profile of the IgG subclasses for the various FcγR types. Such differences are evident in the IgG subclasses from all mammalian species and along with the composition of the Fc-associated glycan, represent the main determinants for regulating Fc-FcγR interactions.

In all mammalian species, IgG molecules share highly conserved structural characteristics that determine the capacity of the Fc domain to interact with the various FcγRs and regulate its affinity. As FcγRs have co-evolved with IgG molecules during the emergence of the mammalian class, they feature a number of highly conserved properties that are fundamental for their ligand binding and signaling activities. For example, type I FcγRs can transduce either activating or inhibitory signals upon engagement by the IgG Fc domain, which is accomplished through specialized signaling motifs at their intracellular domains (Bournazos et al. 2017). Activating type I FcγRs feature intracellular tyrosine activating motifs (ITAMs) at their cytoplasmic domains, which transduce cell activating signals upon receptor crosslinking by the Fc domains of IgG immune complexes. Such signaling motifs are highly conserved among mammalian species and several innate and adaptive immunoreceptors, including TCRs, depend on ITAM-mediated signaling for their function. Activating type I FcγR-mediated signaling is counterbalanced by inhibitory FcγRs, whose cytoplasmic domains comprise intracellular tyrosine inhibition motifs (ITIMs) that associate with tyrosine phosphatases to inhibit kinase activity. The balancing activity of ITAM- and ITIM-containing FcγRs represents a key homeostatic mechanism that controls IgG-mediated inflammation and limits excessive or inappropriate cellular activation. Indeed, in nearly all effector leukocytes, activating type I FcγR expression is coupled with the expression of an inhibitory FcγR that regulates the signaling outcome of Fc-FcγR interactions. In addition to FcγRs, paired co-expression of activating and inhibitory immunoreceptors is very commonly found in several immune pathways, representing a

fundamental mechanism for the control a number of processes of the innate and adaptive immunity.

In addition to the intracellular motifs and the downstream components that are required for the signal transduction of FcγRs, the FcγR ligand binding domains share highly homologous features among mammalian species. All type I FcγRs are members of the IgSF and their extracellular, IgG-binding domain consists of two (or three for the high-affinity FcγRI) C2-type IgSF domains. On the contrary, type II FcγRs are not structurally related to type I FcγRs, as their extracellular domain belongs to the C-type lectin family of receptors. Consistent with the structural differences in their extracellular, ligand-binding domains, type I and type II FcγRs engage the IgG Fc domain at distinct, non-overlapping regions; the hinge-proximal region of CH2 serves as the binding site for type I FcγRs, whereas type II FcγRs engage the Fc domain at the CH2–3 interface in a 2:1 (receptor:IgG) binding stoichiometry (Sondermann et al. 2013). In humans, several genes, each with multiple transcriptional isoforms, encode the different type I and type II FcγRs (Pincetic et al. 2014). Consistent with their common functional properties, members of the type I and type II FcγRs are mapped at specific loci on human chromosome 1 (1q23) and 19 (19p13), respectively. Clustering of the various human FcγR genes at specific genomic loci, along with the high degree of sequence homology between members of the same FcγR type, suggest that mammalian FcγR genes have emerged from a common ancestral precursor gene. Indeed, comparative analysis of the type I FcγR gene locus among different mammalian species shows a high degree of sequence homology, indicative of the emergence of this locus very early in mammalian evolutionary history (Fig. 1). This locus consists of three type I FcγR genes, corresponding to the human *FCGR2A*, *FCGR3A*, and *FCGR2B* genes. The genomic organization of this locus is conserved among diverse mammalian species, from very primitive mammals to primates, except for humans and chimpanzees. Due to the high sequence similarity of the ancestral *FCGR2A* and *FCGR2B* genes, sequential non-homologous recombination events that occurred late in human and chimpanzee divergence from the common non-human primate ancestor generated additional type I FcγR genes (Qiu et al. 1990) (Fig. 2). These genes are uniquely found in humans and chimpanzees and include *FCGR2C* and *FCGR3B*, which encode for FcγRIIc and FcγRIIIb, respectively. *FCGR3B* originates from a gene duplication event of *FCGR3A* and both genes share a very high degree of sequence homology. However, contrary to FcγRIIIa, FcγRIIIb is processed as a GPI-anchored molecule and lacks an intracellular domain; an effect attributed to the presence of a single nucleotide substitution (F203S) within the *FCGR3B* coding sequence. This point mutation is mapped at the membrane proximal region of the extracellular domain of FcγRIIIb and creates a post-translational modification signal sequence that processes FcγRIIIb as a GPI-anchored protein. Another type I FcγR gene that is uniquely found in humans and chimpanzees is *FCGR2C*, which is the result of the non-homologous recombination between the *FCGR2A* and *FCGR2B* genes. *FCGR2C* is essentially a

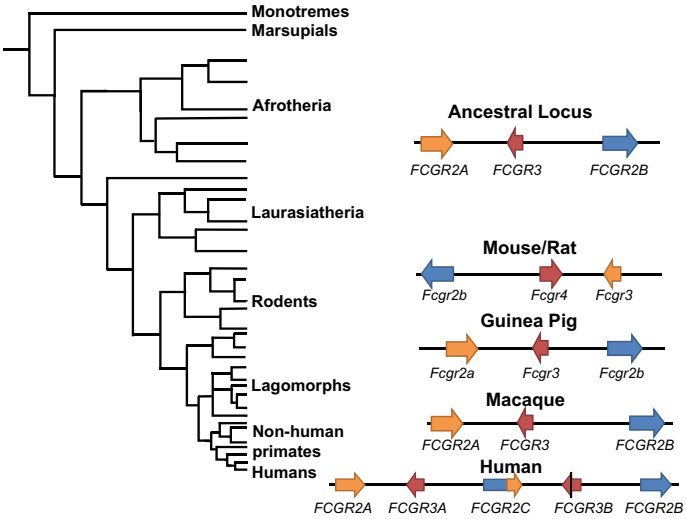


**Fig. 1** Evolutionary conservation of the FcγR locus among mammalian species. The ancestral FcγR locus comprises three low-affinity FcγR genes that encode two activating (*FCGR2A* and *FCGR3*) and one inhibitory FcγRs (*FCGR2B*). These FcγRs feature two extracellular C2-type IgSF domains that mediate the recognition of the IgG Fc domain. This ancestral locus shares a common genomic structure and exhibits high sequence homology in diverse mammalian species, including non-human primates, like rhesus macaques. However, in humans and chimpanzees, the FcγR locus features unique characteristics and comprises additional FcγR genes (*FCGR2C* and *FCGR3B*)

chimeric gene, which comprises the exons that encode the extracellular domain of FcγRIIb, whereas the transmembrane and intracellular domains originate from FcγRIIa.

## 3 Inter-species Differences and Limitations for the Study of Human FcγR Function

Differences in the FcγR biology between humans and other mammalian species are not limited to the unique organization of the human FcγR locus and the presence of additional FcγR genes, like *FCGR3B* and *FCGR2C*, but extend to the FcγR structural characteristics and expression pattern among the various leukocyte cell types. For example, in murine species, all activating FcγRs require the FcR γ-chain for expression, assembly to the cell membrane, and signaling, whereas, in humans, FcγRIIa and FcγRIIc expression and signaling is not dependent on FcR γ-chain expression, as the cytoplasmic domains of these FcγRs contain ITAMs that can sufficiently transduce signals upon receptor engagement. FcγRIIIb, which is exclusively expressed in humans, has a unique structure that is not found in any